

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/68	A2	(11) International Publication Number: WO 98/02582 (43) International Publication Date: 22 January 1998 (22.01.98)
<p>(21) International Application Number: PCT/US97/12347</p> <p>(22) International Filing Date: 15 July 1997 (15.07.97)</p> <p>(30) Priority Data: 60/021,818 16 July 1996 (16.07.96) US</p> <p>(71) Applicant: GEN-PROBE INCORPORATED [US/US]; 10210 Genetic Center Drive, San Diego, CA 92121 (US).</p> <p>(72) Inventors: BECKER, Michael, McClellan; 12819 Meadowdale Lane, San Diego, CA 92131 (US). MAJLESSI, Mehrdad; 15895 Sunnyfield Place, San Diego, CA 92127 (US).</p> <p>(74) Agents: HEBER, Sheldon, O. et al.; Lyon & Lyon LLP, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).</p>		<p>(81) Designated States: AU, CA, JP, KR, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published <i>Without international search report and to be republished upon receipt of that report.</i></p>
<p>(54) Title: METHODS FOR DETECTING AND AMPLIFYING NUCLEIC ACID SEQUENCES USING MODIFIED OLIGONUCLEOTIDES HAVING INCREASED TARGET SPECIFIC T_M</p> <p>(57) Abstract</p> <p>The present invention concerns oligonucleotides containing one or more modified nucleotides which increase the binding affinity of the oligonucleotides to target nucleic acids having a complementary nucleotide base sequence. These modified oligonucleotides hybridize to the target sequence at a faster rate than unmodified oligonucleotides having an identical nucleotide base sequence. Such modified oligonucleotides include oligonucleotides containing at least one 2'-O-methylribofuranosyl moiety joined to a nitrogenous base. Oligonucleotides can be modified in accordance with the present invention to preferentially bind RNA targets. The present invention also concerns methods of using these modified oligonucleotides and kits containing the same.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

METHODS FOR DETECTING AND AMPLIFYING NUCLEIC ACID SEQUENCES
USING MODIFIED OLIGONUCLEOTIDES HAVING INCREASED TARGET
SPECIFIC T_M

0

Field of the Invention

This invention pertains to methods and compositions for detecting and amplifying nucleic acid sequences using oligonucleotides which contain one or more nucleotides having a
5 modification or modifications resulting in increased target affinity. Such oligonucleotides have been unexpectedly discovered to hybridize to a target nucleic acid at a significantly greater rate than a corresponding unmodified oligonucleotide hybridizes to the same target. As a result, the methods and compositions of the present invention offer advantages for applications employing nucleic acid hybridization, such as medical and veterinary diagnostics,
10 food testing and forensics.

Background of the Invention

In recent years, nucleic acid hybridization has become an increasingly important means
15 of identifying, measuring and detecting the presence of particular nucleic acids in a given sample. Thus, for example, the fields of medical diagnostics, environmental and food testing, and forensics have all benefitted from the use of nucleic acid hybridization as a rapid, simple and extraordinarily accurate way of testing for the presence or absence of given biological contaminants or microorganisms in a sample.

20 Most nucleic acid hybridization schemes have features in common. One such typical feature is the use of single-stranded nucleic acid probes (or denatured double-stranded probes) having a defined or known nucleotide sequence. Probe molecules may be derived from biological sources, such as genomic DNA or RNA, or may be enzymatically synthesized, either in a prokaryotic or eukaryotic host cell or in vitro. Presently, most nucleic
25 acid probes in common use are oligonucleotide probes made using chemical synthetic methods ("synthetic oligonucleotides"). One such synthetic method is automated sequential

addition of 3'-activated, protected nucleotides to the 5' end of a growing, solid phase-bound oligonucleotide chain, followed by cleavage of the completed oligonucleotide from the support and deprotection. See, e.g., Eckstein, Oligonucleotides & Analogues: A Practical Approach (1991).

5 Synthetic oligonucleotides for use as hybridization probes are typically deoxyribonucleotides having a nucleotide sequence complementary to a nucleotide sequence of the nucleic acid to be detected. DNA oligonucleotides are classically preferred for a number of reasons. Among these is the greater stability DNA has to enzymatic hydrolysis upon exposure to common samples, due to the almost ubiquitous presence in samples of
10 various RNases. RNA is also known to be less chemically stable than DNA, e.g., RNA degradation is facilitated by the presence of base, heavy metals. And compared to RNA, DNA is less prone to assume stable secondary structures under assay conditions. Such secondary structures can render oligonucleotides unavailable for inter-molecular hybridization. Nevertheless, RNA oligonucleotides may be used, even though they are less preferred.

15 Nucleic acid hybridization exploits the ability of single-stranded nucleic acids to form stable hybrids with corresponding regions of nucleic acid strands having complementary nucleotide sequences. Such hybrids usually consist of double-stranded duplexes, although triple-stranded structures are also known. Generally speaking, single-strands of DNA or RNA are formed from nucleotides containing the bases adenine (A), cytosine (C), thymidine (T),
20 guanine (G), uracil (U), or inosine (I). The single-stranded chains may hybridize to form a double-stranded structure held together by hydrogen bonds between pairs of complementary bases. Generally, A is hydrogen bonded to T or U, while G or I is hydrogen bonded to C. Along the double-stranded chain, classical base pairs of AT or AU, TA or UA, GC, or CG are present. Additionally, some mismatched base pairs (e.g., AG, GU) may be present. Under
25 appropriate hybridization conditions, DNA/DNA, RNA/DNA, or RNA/RNA hybrids can form.

By "complementary" is meant that the nucleotide sequences of corresponding regions of two single-stranded nucleic acids, or two different regions of the same single-stranded nucleic acid, have a nucleotide base composition that allows the single strands to hybridize

together in a stable double-stranded hydrogen-bonded region under stringent hybridization conditions. When a contiguous sequence of nucleotides of one single stranded region is able to form a series of "canonical" hydrogen-bonded base pairs with an analogous sequence of nucleotides of the other single-stranded region, such that A is paired with U or T, and C is paired with G, the nucleotides sequences are "perfectly" complementary.

The extreme specificity of nucleic acid hybridization, which under some circumstances can allow the discrimination of nucleic acids differing by as little as one base, has allowed the development of hybridization-based assays of samples containing specific microorganisms, nucleic acids bearing given genetic markers, tissue, biological fluids and the like. Such assays are often able to identify nucleic acids belonging to particular species of microorganisms in a sample containing other, closely-related species. Nucleic acid hybridization assays can also specifically detect or identify certain individuals, or groups of individuals, within a species, such as in the forensic use of RFLP (restriction fragment length polymorphism) and PCR (polymerase chain reaction) testing of samples of human origin.

The use of oligonucleotides as a diagnostic tool in nucleic acid hybridization testing often involves, but need not involve, the use of a reporting group or "label" which is joined to the oligonucleotide probe molecule, or both the probe and the target. Such a reporter group moiety may include, for example, a radioisotope, chemiluminescent or fluorescent agent, or enzyme joined to the oligonucleotide. The label is employed to render the probe capable of detection, particularly when the probe is hybridized to the target nucleic acid.

The majority of assay methods employing nucleic acids utilize a physical separation step in order to separate the probe:analyte hybrid from unbound probe. These assay methods are called "heterogeneous" assays. In nucleic acid hybridization assays, an analyte molecule is the target nucleic acid species sought to be detected, quantitated and/or identified. A "hybrid" is a partly or wholly double-stranded nucleic acid comprising two single-stranded nucleic acids, such as a probe and a target nucleic acid, having a region of complementarity resulting in intermolecular hydrogen bonding under assay and/or amplification conditions.

Assay methods utilizing a physical separation step include methods employing a solid-

phase matrix, such as glass, minerals or polymeric materials, in the separation process. The separation may involve preferentially binding the probe:analyte complex to the solid phase matrix, while allowing the unassociated probe molecules to remain in a liquid phase. Such binding may be non-specific, as, for example, in the case of hydroxyapatite, or specific, for example, through sequence-specific interaction of the target nucleic acid with a "capture" probe which is directly or indirectly immobilized on the solid support. In any such case, the amount of probe remaining bound to the solid phase support after a washing step is proportional to the amount of analyte in the sample.

Alternatively, the assay may involve preferentially binding the unhybridized probe while leaving the hybrid to remain in the liquid phase. In this case the amount of probe in the liquid phase after a washing step is proportional to the amount of analyte in the original sample. When the probe is a nucleic acid or oligonucleotide, the solid support can include, without limitation, an adsorbent such as hydroxyapatite, a polycationic moiety, a hydrophobic or "reverse phase" material, an ion-exchange matrix, such as DEAE, a gel filtration matrix, or a combination of one or more of these solid phase materials. The solid support may contain one or more oligonucleotides, or other specific binding moiety, to capture, directly or indirectly, probe, target, or both. In the case of media, such as gel filtration, polyacrylamide gel or agarose gel, the separation is not due to binding of the oligonucleotide but is caused by molecular sieving of differently sized or shaped molecules. In the latter two cases, separation may be driven electrophoretically by application of an electrical current through the gel causing the differential migration through the gel of nucleic acids of different sizes or shapes, such as double-stranded and single-stranded nucleic acids.

A heterogeneous assay method may also involve binding the probe to a solid-phase matrix prior to addition of a sample suspected of containing the analyte of interest. The sample can be contacted with the label under conditions which would cause the desired nucleic acid to be labeled, if present in the sample mixture. The solid phase matrix may be derivatized or activated so that a covalent bond is formed between the probe and the matrix. Alternatively, the probe may be bound to the matrix through strong non-covalent interactions,

including, without limitation, the following interactions: ionic, hydrophobic, reverse-phase, immunobinding, chelating, and enzyme-substrate. After the matrix-bound probe is exposed to the labeled nucleic acid under conditions allowing the formation of a hybrid, the separation step is accomplished by washing the solid-phase matrix free of any unbound, labeled analyte.

5 Conversely, the analyte can be bound to the solid phase matrix and contacted with labeled probe, with the excess free probe washed from the matrix before detection of the label.

Yet another type of assay system is termed "homogeneous assay." Homogeneous assays can generally take place in solution, without a solid phase separation step, and commonly exploit chemical differences between the free probe and the analyte:probe
10 complex. An example of an assay system which can be used in a homogeneous or heterogeneous format is the hybridization protection assay (HPATM) disclosed in Arnold, et al., U.S. Patent 5,283,174, in which a probe is linked to a chemiluminescent moiety, contacted with an analyte and then subjected to selective chemical degradation or a detectable change in stability under conditions which alter the chemiluminescent reagent bound or joined to
15 unhybridized probe, without altering the chemiluminescent reagent bound or joined to an analyte:probe conjugate. Subsequent initiation of a chemiluminescent reaction causes the hybrid-associated label to emit light. This patent enjoys common ownership with the present application and is expressly incorporated by reference herein.

Competition assays, in which a labeled probe or analyte competes for binding with its
20 unlabeled analog, are also commonly used in a heterogeneous format. Depending on how the system is designed, either the amount of bound, labeled probe or the amount of unbound, labeled probe can be correlated with the amount of analyte in a sample. However, such an assay can also be used in a homogeneous format without a physical separation step, or in a format incorporating elements of both a homogeneous and a heterogeneous assay.

25 The assay methods described herein are merely illustrative and should not be understood as exhausting the assay formats employing nucleic acids known to those of skill in the art.

Nucleic acid hybridization has been utilized in methods aimed at using

oligonucleotides as therapeutic agents to modify or inhibit gene expression within living organisms. In an example of such utilization, oligonucleotide "antisense" agents can be targeted specifically to an mRNA species encoding a deleterious gene product, such as a viral protein or an oncogene. See, e.g., Zamecnik and Stephenson, 75 Proc. Nat'l Acad. Sci. (USA), 280-284 (1978); Stephenson and Zamecnik, 75 Proc. Nat'l Acad. Sci. (USA), 285-288 (1978); and Tullis, U.S. Patent No. 5,023,243. Although Applicant does not wish to be bound by theory, it is thought that the RNA:DNA duplex which results from the binding of the antisense oligonucleotide to RNA targets may serve as a substrate for RNase H, an RNA-degrading enzyme present in most cells and specific for RNA contained in an RNA:DNA duplex. According to this model, the target RNA molecule is destroyed through hybridization to the antisense oligonucleotide. Variations of this general strategy exist, wherein, for example, the oligonucleotide has a structure conferring an enzymatic activity on the oligonucleotide, such as the RNase activity of so-called ribozymes. See, e.g., Goodchild, PCT Publication No. WO93/15194.

Because therapeutic antisense oligonucleotides are primarily designed to function in vivo, formulations for the delivery of such agents must not significantly inhibit normal cellular function. Thus, nuclease inhibitors, which can sometimes be included in in vitro diagnostic tests to prevent oligonucleotide degradation, are not suitable for use in vivo. This fact has resulted in the design of various oligonucleotides modified at the internucleotide linkage, at the base or sugar moieties, or at combinations of these sites to have greater nuclease resistance than unmodified DNA.

Thus, a number of oligonucleotide derivatives have been made having modifications at the nitrogenous base, including replacement of the amino group at the 6 position of adenosine by hydrogen to yield purine; substitution of the 6-keto oxygen of guanosine with hydrogen to yield 2-amino purine, or with sulphur to yield 6-thioguanosine, and replacement of the 4-keto oxygen of thymidine with either sulphur or hydrogen to yield, respectively, 4-thiothymidine or 4-hydrothymidine. All these nucleotide analogues can be used as reactants for the synthesis of oligonucleotides. See, e.g., Oligonucleotides and Analogues: A Practical Approach, supra.

Other substituted bases are known in the art. See, e.g., Cook, et al., PCT Publication No. WO92/02258, entitled Nuclease Resistant, Pyrimidine Modified Oligonucleotides that Detect and Modulate Gene Expression, which is incorporated by reference herein. Base-modified nucleotide derivatives can be commercially obtained for oligonucleotide synthesis.

5 Similarly, a number of nucleotide derivatives have been reported having modifications of the ribofuranosyl or deoxyribofuranosyl moiety. See, e.g., Cook et al., PCT Publication No. WO94/19023 entitled Cyclobutyl Antisense Oligonucleotides, Methods of Making and Use Thereof; McGee, et al., PCT Publication No. WO94/02501 entitled Novel 2'-O-Alkyl Nucleosides and Phosphoramidites Processes for the Preparation and Uses Thereof; and Cook,
10 PCT Publication No. WO93/13121 entitled Gapped 2'-modified Oligonucleotides. These three publications are incorporated by reference herein.

Most oligonucleotides comprising such modified bases have been formulated with increased cellular uptake, nuclease resistance, and/or increased substrate binding in mind. In other words, such oligonucleotides are described as therapeutic gene-modulating agents.

15 Nucleic acids having modified nucleotide residues exist in nature. Thus, depending on the type or source, modified bases in RNA can include methylated or dimethylated bases, deaminated bases, carboxylated bases, thiolated bases and bases having various combinations of these modifications. Additionally, 2'-O-alkylated bases are known to be present in naturally occurring nucleic acids. See, Adams, The Biochemistry of the Nucleic Acids, 7,8
20 (11th ed. 1993).

Summary of the Invention

25 This invention concerns diagnostic methods and compositions employing nucleic acid hybridization techniques. Applicant has surprisingly discovered that oligonucleotides, comprised of one or more modified nucleotides, which have increased binding affinity to a target nucleic acid having a complementary nucleotide sequence, will hybridize to the target nucleic acid at a faster rate than unmodified oligonucleotides. The inventions described

herein are drawn to the use of oligonucleotides, wholly or partially so modified, in methods involving their use as, for example, hybridization assay probes, amplification primers, helper oligonucleotides, and oligonucleotides for the capture and immobilization of desired nucleic acids.

5 Although the present invention is not to be seen as so limited, in particularly preferred embodiments the present invention concerns diagnostic methods utilizing oligonucleotides having nucleotides with 2' modifications to their ribofuranosyl (or deoxyribofuranosyl) moieties. In particular, Applicant has discovered that incorporating nucleotides having such modifications as part of synthetic oligonucleotides can profoundly increase the rate of nucleic acid hybridization, and the preferential binding of such oligonucleotides, to RNA targets over DNA targets. A currently preferred embodiment makes use of oligonucleotides containing nucleotide analogues having 2'-O-methylribofuranosyl moieties linked to a nitrogenous base. Other substitutions at the 2' position of the sugar would be expected, in light of the present disclosure, to have similar properties so long as the substitution is not so large as to cause
10 steric inhibition of hybridization.
15

 Additionally, in light of the discoveries giving rise to the present invention, other modifications which increase the T_m of a modified oligonucleotide:target hybrid would reasonably be expected to contribute to increases in the rate of hybridization as well. Such modifications may occur at the 2' position (or other positions) of the deoxyribofuranosyl or ribofuranosyl moiety (such as 2' halide substitutions), on the nitrogenous bases (such as N-diisobutylaminomethylidene-5-(1-propynyl)-2'-deoxycytidine; a cytidine analog, or 5-(1-propynyl)-2'-deoxyuridine); a thymidine analog, or in the linkage moiety. Thus, while specific reference is made throughout this application to 2' modifications, those of skill in the art will understand that other modifications leading to an increased T_m of a modified
20 oligonucleotide:target hybrid over a hybrid containing an unmodified oligonucleotide of identical base sequence would be expected to have similar properties and effects on hybridization kinetics.
25

 While the term T_m refers to the temperature at which 50% of a population of equal

amounts of complementary nucleic acid strands are in the double-stranded form, throughout this disclosure the " T_m of an oligonucleotide" or a nucleic acid (single-stranded) is intended to mean the T_m of an oligonucleotide or nucleic acid in a nucleic acid duplex with a target, wherein the nucleic acid contains a base sequence region which is exactly complementary to a base sequence region of the oligonucleotide, unless otherwise indicated.

Because the T_m of the modified oligonucleotides is higher than that of corresponding, unmodified oligonucleotides of the same base sequence, the compositions and diagnostic methods described herein enable the use of oligonucleotides and oligonucleotide probes of shorter length than are otherwise practical for the specific hybridization and detection of nucleic acid targets (preferably RNA targets). The use of shorter oligonucleotides to specifically bind to target nucleic acids at a given temperature has additional advantages. For instance, shorter oligonucleotides will generally have a greater ability to discriminate perfectly complementary targets from "mismatched" base sequence regions. Shorter oligonucleotides are also less likely to overlap undesirable base sequences. Additionally, because of the higher T_m , the modified oligonucleotides can stably hybridize at higher temperatures than their unmodified counterparts.

The use of higher hybridization temperatures kinetically drives the hybridization reaction, resulting in faster hybridization rates than would occur at lower temperatures. Further, the modified oligonucleotides used in the methods of the present invention result in faster hybridization rates than the unmodified versions, even when the temperature is not raised.

An increased hybridization rate leads to several other advantages in diagnostic assays. For example, diagnostic assays conducted in accordance with the present invention can be conducted more rapidly than in previously existing hybridization assays. In cases in which the results of the assay may dictate a course of medical treatment or other action, a faster assay result has clear prognostic advantages and may result in more effective treatment. Also, owing to faster hybridization rates and greater affinity of modified oligonucleotides for targets, especially RNA targets, lower concentrations of probe may be used to achieve the

same amount of signal. Thus, the assay background (or "noise") can be reduced and the lower concentration of probe can help eliminate undesirable cross-reactions with non-target nucleic acids. Additionally, these assays may be run in larger volumes of sample, thus increasing the sensitivity of the assay.

5 Thus, hybridization assay probes, amplification oligonucleotides, sample preparation oligonucleotides and/or helper oligonucleotides can all be designed to contain modified bases which have the advantage of increasing the rate of target-specific hybridization.

Brief Description of the Drawings

10 Figure 1 provides the IUPAC nomenclature for a sampling of acridinium esters that may be used as detectable chemiluminescent labels in the present disclosure.

15 Figure 2 shows an arrangement whereby detection of an analyte first requires the hybridization of the analyte to a nucleic acid other than the probe. According to this arrangement, the probe is unable to bind with either the analyte or the non-probe nucleic acid before the analyte has hybridized to the non-probe nucleic acid. (Bolded portions represent regions of complementary between the analyte and the non-probe nucleic acid.) However, hybridization of the analyte to the non-probe nucleic acid alters the configuration of the non-probe nucleic acid sufficiently to enable hybridization of the non-probe nucleic acid to the probe, thus permitting detection of the analyte.

20 Figure 3 shows the melting curve of a 2'-O-methyl oligonucleotide probe with either an RNA target or a DNA target (two independent experiments), where melting is shown as an increase in light absorbance at 260nm (hyperchromatic shift).

Figure 4 shows hybridization of a single concentration of acridinium ester-labeled deoxy- or 2'-O-methyl oligonucleotides of identical base sequence to varying amounts of a

fully complementary RNA target during a fixed time of hybridization.

Figure 5 shows hybridization of varying amounts of acridinium ester-labeled deoxy- or 2'-O-methyl oligonucleotides of identical base sequence to fixed amounts of a fully complementary RNA target during a fixed time of hybridization.

Figure 6 shows hybridization of a fixed amount of acridinium ester-labeled deoxy- and 2'-O-methyl oligonucleotides of identical base sequence to a fixed amount of a fully complementary RNA target for various times of hybridization.

Figure 7 shows hybridization of a DNA or 2'-O-methyl oligonucleotide probe to a fully complementary RNA target. The data are plotted according to the equation $\ln(1-H) = (k)(C_0t)$, where H is the percent hybridization, k is the hybridization rate constant, C_0 is the concentration of probe, and t is time.

Detailed Description of the Invention

Definitions

Unless clearly indicated otherwise, the following terms will have the indicated meanings throughout this specification.

By "nucleic acid analyte" or "analyte" is meant a nucleic acid sought to be detected in a sample or a nucleic acid synthesized as a result of a nucleic acid amplification reaction which contains at least about 20 nucleotides of the nucleotide base sequence of a nucleic acid sought to be detected in a sample or the complement thereof.

By "synthesizing" a nucleic acid or oligonucleotide is meant making the nucleic acid by chemical synthesis or enzymatic means. It is known that certain nucleic acid polymerase

enzymes can incorporate modified nucleotides during enzymatic synthesis.

By "modified", a "modified nucleotide" or "modification" is meant a purposeful variant from the classical ribo- and deoxyribonucleotides A, T, G, C and U. When used in this specification, modified will mean a variant of the classical nucleotides, said variants leading to a higher binding efficiency when an oligonucleotide which contains said modified nucleotides is hybridized to a target nucleic acid than when the same oligonucleotide contains the classical nucleotides. In some cases an oligonucleotide having a modified 3' end may be referred to. This means that the 3' end of the oligonucleotide contains a substitution which inhibits or prevents extension of the 3' end by a nucleic acid polymerase.

By "conjugate molecule" is meant a molecule that can couple with an oligonucleotide in such a way that at least some of the characteristics of both the molecule and the oligonucleotide are retained in the combined product. Most often, the conjugate molecule contributes a new physical or chemical property to the oligonucleotide, while the oligonucleotide retains its ability to base pair.

By "binding affinity" is meant a measure of the strength of hydrogen bonding between at least partly complementary nucleic acids under defined nucleic acid hybridization conditions. A convenient measure of binding efficiency is the T_m , which is the temperature at which 50% of said two strands are in the double-stranded or hybridized form.

By "label" is meant a reporter moiety which is capable of being detected as an indication of the presence of the oligonucleotide to which it is joined. When the labeled oligonucleotide is hybridized to one or more other oligonucleotides, the presence of the label can be an indication of the presence of the other oligonucleotide or oligonucleotides as well. Appropriate reporter moieties are well known in the art and include, for example, radioisotopes, dyes, chemiluminescent, fluorescent, chemiluminescent and electrochemiluminescent compounds, nucleic acid sequences, enzymes, enzyme substrates, chromophores and haptens.

By "nucleic acid assay conditions" is meant environmental conditions, including temperature and salt concentration, for the preferential formation of stable hybrids between

complementary base sequence regions over the formation of stable hybrids between non-complementary base sequence regions.

By "acridinium ester derivative" or "AE" is meant any of a family of chemiluminescent compounds derived from the acridinium ring which have a labelled ester or ester-like linkage at the C9 position connecting the acridinium ring to a leaving group. The leaving group is preferably an aryl or substituted aryl group. Substitutions, such as alkyl (e.g., methyl), alkoxy (e.g., methoxy), aryl and halide (e.g., Br and F), may be made to either or both the acridinium ring or the leaving group. Examples of such acridinium esters are provided in Figure 1.

The methods and compositions of the present invention result from the unexpected discovery that oligonucleotides containing one or more nucleotides modified so that the oligonucleotides have an increased T_m for a given target (as compared to otherwise identical unmodified oligonucleotides) will hybridize to a given target at an increased rate as compared to unmodified oligonucleotides. A maximum increase in the hybridization rate of a modified oligonucleotide occurs when a "cluster" of nucleotides are modified. By "cluster" is meant that at least about 4 of 5 consecutive nucleotides are so modified. Thus, oligonucleotides containing a mixture of modified and unmodified nucleotides may be just as effective in increasing target hybridization rate as in oligonucleotides containing 100% modified nucleotides. Aspects of the invention feature "chimeric" oligonucleotides containing both modified and unmodified nucleotides.

When used in this context, a "target" nucleic acid is a nucleic acid sought to be hybridized with an oligonucleotide. Such a nucleic acid may be a naturally occurring nucleic acid, e.g., ribosomal RNA, it may be the product (i.e., an "amplicon") of nucleic acid amplification methods such as PCR or a transcription-based amplification method, as described more fully below, or it may be another synthetic oligonucleotide.

Thus, the present invention is directed to diagnostic methods and compositions involving modified oligonucleotides which display an increase in the rate of oligo:target

hybridization over an unmodified oligonucleotide of the same base sequence.

In a preferred embodiment, the invention utilizes modifications to the 2' position of the deoxyribofuranosyl (or ribofuranosyl) ring. The 2'-modification involves the placement of a group other than hydrogen or hydroxyl at the 2' position of the ribofuranosyl ring.

5 Regardless of the nature of the substitution, it must not sterically hinder the ability of an oligonucleotide containing one or more such nucleotide modifications to hybridize to a single-stranded oligonucleotide having a complementary nucleotide base sequence. The hybridization of complementary, double-stranded nucleic acids in which one strand contains such modifications is markedly increased as compared to situations in which neither strand is
10 so modified.

When a modified oligonucleotide is referred to as having an "increased" or "greater" affinity or rate, it is meant that the rate of hybridization or affinity of the modified oligonucleotide is greater than the hybridization rate or binding affinity of an unmodified oligonucleotide of the same length and base sequence to the same target.

15 Particularly preferred oligonucleotides are substituted with a methoxy group at the 2' sugar position. These 2'-modified oligonucleotides display a preference for RNA over DNA targets having a sequence identical to the RNA target (but having T substituted for U), with respect to both T_m and hybridization kinetics. Other such oligonucleotides are known in the art.

20 Conjugate molecules attached to oligonucleotides modified as described herein may function to further increase the binding affinity and hybridization rate of these oligonucleotides to a target. Such conjugate molecules may include, by way of example, cationic amines, intercalating dyes, antibiotics, proteins, peptide fragments, and metal ion complexes. Common cationic amines include, for example, spermine and spermidine, i.e.,
25 polyamines. Intercalating dyes known in the art include, for example, ethidium bromide, acridines and proflavine. Antibiotics which can bind to nucleic acids include, for example, actinomycin and netropsin. Proteins capable of binding to nucleic acids include, for example, restriction enzymes, transcription factors, and DNA and RNA modifying enzymes. Peptide

fragments capable of binding to nucleic acids may contain, for example, a SPKK (serine-proline-lysine (arginine)-lysine (arginine)) motif, a KH motif or a RGG (arginine-glycine-glycine) box motif. See, e.g., Suzuki, EMBOJ, 8:797-804 (1989); and Bund, et al., Science, 265:615-621 (1994). Metal ion complexes which bind nucleic acids include, for example, cobalt hexamine and 1,10-phenanthroline-copper. Oligonucleotides represent yet another kind of conjugate molecule when, for example, the resulting hybrid includes three or more nucleic acids. An example of such a hybrid would be a triplex comprised of a target nucleic acid, an oligonucleotide probe hybridized to the target, and an oligonucleotide conjugate molecule hybridized to the probe. Conjugate molecules may bind to oligonucleotides by a variety of means, including, but not limited to, intercalation, groove interaction, electrostatic binding, and hydrogen bonding. Those skilled in the art will appreciate other conjugate molecules that can be attached to the modified oligonucleotides of the present invention. See, e.g., Goodchild, Bioconjugate Chemistry, 1(3):165-187 (1990). Moreover, a conjugate molecule can be bound or joined to a nucleotide or nucleotides either before or after synthesis of the oligonucleotide containing the nucleotide or nucleotides.

Applicant has also unexpectedly discovered that the observed increase in hybridization rate of modified oligonucleotides to their targets does not always increase indefinitely with an increasing number of contiguous modified nucleotides, especially when the target possesses an open or unfolded structure, or when helper probes are present. Under such circumstances, placement of modified nucleotides in a substantially contiguous arrangement, i.e., about 4 of 5 contiguous nucleotides, in the oligonucleotide, followed by hybridization to a complementary target, will result in an increased hybridization rate only up to a given number of modifications. The addition of modified nucleotides to the cluster past this number will not, in general, substantially further increase the hybridization rate.

In a currently preferred modification, employing 2'-O-methyl-substituted nucleotides, optimal hybridization rates are obtained in oligonucleotides having a cluster of about 8 contiguous modified residues. Given the discovery that modified oligonucleotides can increase the hybridization rate, it was unexpected that this effect does not always parallel the

increase in T_m contributed by such additions. That is, addition of modified oligonucleotides past the rate-optimal cluster size will continue to increase the T_m .

Although Applicant does not wish to be limited by theory, it is believed that such clusters function as "nucleation centers" which are the first regions of the oligonucleotide or nucleic acid to hydrogen-bond, in a rate-limiting step, followed by rapid hydrogen bonding of the remaining bases. While the entire oligonucleotide or nucleic acid may be so modified, little advantage in increased hybridization rate appears to be gained by substantially exceeding the optimal cluster size.

However, when the structure of the target is closed or folded in nature, and no helper probes are included, the hybridization rate between the oligonucleotide and the target generally may be improved by adding modified nucleotides to the oligonucleotide in excess of about 4 contiguous nucleotides. In a preferred embodiment, substantially all of the nucleotides of an oligonucleotide complementary to the structurally closed target will be modified.

The rate of in-solution hybridization of two complementary single-stranded nucleic acids depends on various factors, such as the concentration of the nucleic acids, the temperature of hybridization, and the properties of the solvent solution, such as salt concentration. Various methodologies have been employed to increase hybridization rates, the majority of which involve either changing the solvent system, such as by forming emulsions of immiscible solvents; by employing nucleic acid precipitating agents (e.g., Kohne et al., U.S. Patent No. 5,132,207), or volume exclusion agents, such as polyethylene glycol; or by increasing the concentration of a nucleic acid strand.

A problem associated with the latter approach in a diagnostic assay is that the target nucleic acid is usually present in quite small amounts. Thus, to increase the nucleic acid concentration necessitates using an excess of the oligonucleotide, resulting in increased cost and reagent waste and, if the oligonucleotide is labeled, risking unacceptably high backgrounds. The other methods, such as those requiring the use of multiple solvents and agents, such as polyethylene glycol, may present practical difficulties, such as excessive

sample manipulation and time.

Therefore, one aspect of the present invention provides a means for increasing hybridization rates, as well as binding affinity, of oligonucleotides for RNA targets by using oligonucleotides containing nucleotides having a substitution at the 2' position of the ribofuranosyl ring ("2'-modified oligonucleotide"); preferably an alkoxy substitution, most preferably a methoxy substitution. These properties render useful methods employing such oligonucleotides in a diagnostic hybridization assay format by increasing the rate and extent of hybridization of such an oligonucleotide without requiring a concomitant increase in the concentrations of the hybridizing nucleic acids, a change in the properties or composition of the hybridization solution, the addition of "helper oligonucleotides", disclosed in Hogan & Milliman, U.S. Patent No. 5,030,557, or an increase in the hybridization temperature. U.S. Patent No. 5,030,557 enjoys common ownership with the present invention and is incorporated by reference herein. Nonetheless, the methods of the present invention may be used as supplements to one or more of these other techniques in any procedure in which an increase in the rate of nucleic acid hybridization would be advantageous.

As mentioned above, an advantage of this aspect of the invention relates to the ability of 2'-O-methyl modified oligonucleotides to preferentially hybridize to RNA over DNA. This property allows the design of oligonucleotide probes targeted to RNA. Probes can be made which would not tend to bind to DNA under stringent hybridization conditions, even where the DNA sequence is identical to the RNA target sequence (except that T is substituted for U in the DNA sequence). Such properties can be used in a number of different formats in which the specific detection of RNA would be advantageous. For example, to indicate and measure changes in the rate of transcription of particular RNA species, such as, for example, specific mRNA species, to monitor the effectiveness of a given therapy, to specifically probe tRNA or rRNA in preference to the genes encoding these RNA species, and to specifically detect RNA viruses in nucleic acid preparations containing large amounts of chromosomal DNA, even DNA preparations containing DNA versions of the viral sequences.

An additional advantage of this and other aspects is an increased target:oligo T_m when

using modified oligonucleotides, such as 2'-modified nucleotides as compared to the T_m of target:oligo hybrids in which the oligonucleotide is a deoxyoligonucleotide. By "target:oligo" is meant a hydrogen bonded, double-stranded nucleic acid complex comprising a single-stranded oligonucleotide. The stability of the target:probe complex increases with an increase
5 in the number of 2'-modified nucleotide residues contained in the probe. By contrast, the increase in the hybridization rate appears to be optimal in nucleic acids having a cluster of about 8 2'-modified nucleotide residues and does not increase significantly upon the addition of consecutive modified oligonucleotide residues above that number. Further, "chimeric" oligonucleotides having at least one such cluster of modified nucleotides could be designed to
10 have greater hybridization rates without necessarily significantly increasing the T_m of the oligonucleotide as a whole to its target.

An increased T_m may be exploited in any diagnostic procedure in which the added stability of a nucleic acid duplex is desired. For example, higher hybridization temperatures can be used to accelerate the hybridization rate. A higher T_m also permits the use of
15 substantially shorter oligonucleotides than were heretofore practical, thus resulting in a savings in the costs associated with producing oligonucleotides for hybridization, as well as other advantages, as mentioned above.

In other aspects and embodiments of the present invention, chimeric oligonucleotides may contain a modified portion designed to bind to target nucleic acid and may also contain a
20 deoxynucleotide portion which is either directly or indirectly able to bind to a solid phase-bound oligonucleotide. By way of example, and not of limitation, such an oligonucleotide may be a target capture oligonucleotide designed to bind a target nucleic acid (e.g., in solution) and link the bound target nucleic acid to a derivatized solid phase matrix, such as a bead, microsphere, polymeric substance, such as agarose or dextran or to a magnetized
25 particle. Derivatives linked to such a matrix may include antibodies, ligands, or wholly or partially single-stranded oligonucleotides having a specific nucleotide sequence, such as a homopolymeric tract, designed to bind the capture oligonucleotide or an intermediate oligonucleotide. The bound target can then be further hybridized with a probe (either RNA,

DNA or modified) and the unbound probe washed free of the immobilized target:probe complex before detecting the presence of the target nucleic acid.

It may be advantageous in certain instances to raise the temperature for hybridizing a modified oligonucleotide to its target. As described above, increasing the hybridization temperature also increases the rate of hybridization, so long as the hybridization temperature is sufficiently below the T_m of the desired hybrid. The hybridization methods claimed herein, employing modified oligonucleotides having a higher T_m than their unmodified counterparts, can be conducted at higher temperatures than would otherwise be used. In such a case, the rate increase associated with the modification alone is further increased by the raised temperature.

What follows are examples of embodiments of the invention, which should not be understood as limiting the scope of the invention thereto. Those skilled in the art will easily comprehend additional embodiments based on the disclosure contained in this specification. Additional embodiments are also contained within the claims which conclude this specification.

Modified Nucleic Acid Hybridization Assay Probes

Nucleic acid hybridization assays utilize one or more nucleic acid probes targeted to, i.e., having a nucleotide base sequence substantially complementary to, a nucleic acid sought to be detected. Often the probe will comprise an oligonucleotide (a single-stranded nucleic acid of between about 10 and about 100 nucleotides in length) which is synthetically made to have a given nucleotide sequence. By "substantially complementary" is meant that the oligo will bind to its target under appropriately selective conditions to form a hydrogen bonded duplex.

While a hybridization assay probe will generally be joined to a detectable label, the probe can be unlabeled and probe:target hybrids detected by, for example, UV absorbance, HPLC chromatography, gel electrophoresis and subsequent staining of the nucleic acid hybrid

or other methods well known in the art. In hybridization assays, the probe and target are contacted with each other under conditions permitting stable and specific hybridization. The resulting hybrid is then separated from unlabeled hybrid and the label detected, or the label can be detected under conditions allowing the detection of the hybrid in preference to the unlabeled probe.

Methods for separating nucleic acids, such as gel exclusion chromatography, reverse phase chromatography, and hydroxyapatite adsorption, are known in the art. Applicant prefers an assay format wherein labeled hybrid and labeled unhybridized probe can be chemically differentiated by virtue of the formation of a double helix. A particularly preferred assay format is the hybridization protection assay (HPA), see U.S. Patent No. 5,283,174 to Arnold et al., in which labeled unhybridized probe can be selectively be made undetectable while hybridized probe is relatively unaffected. Thus, detection of label in this format is an indication of the labeled hybrid.

In these aspects, the present invention involves the use of modified oligonucleotides as probes in a hybridization assay. In one embodiment, modified oligonucleotides having a higher target-specific T_m than unmodified oligonucleotides of the same base sequence are used to increase the rate of hybridization of the assay, as compared to assays employing unmodified oligonucleotides of the same base sequence. Such assay methods are able to utilize higher hybridization temperatures than are practicable using unmodified oligonucleotides. The higher hybridization temperature further increases the rate of hybridization and can also reduce the amount of cross-hybridization (hybridization of the probe with non-target sequences), thereby increasing the specificity of the assay.

The probes of this invention may comprise a cluster of about 4 or more substantially contiguous, modified nucleotide residues mixed with unmodified residues. Alternatively, the probe may comprise 100% modified residues. In preferred embodiments, the modifications are 2' substitutions, such as alkyl, alkoxy and halide substitutions to the 2' carbon of the ribofuranosyl nucleotide moiety. In particularly preferred embodiments, the substitution is a methoxy group.

Particular probe modifications, including 2'-O-methyl substitutions, result in the oligonucleotide having an increased affinity and increased hybridization rate to RNA targets but little effect on DNA affinity or rate of formation of probe:DNA hybrids. Again, this preference to RNA has been found to be optimized when the oligonucleotide has at least one cluster of from about 4 to about 8 modified bases.

Because oligonucleotides modified as described herein have a dramatically increased rate of hybridization, such modified oligonucleotide probes may be used in many cases without the need for the addition of unlabeled "helper probes", which are disclosed in Hogan, supra, as a means for increasing hybridization rates of probe to target. Nevertheless, Applicant has found that in some cases the combined use of such modified oligonucleotides and helper probes may operate in concert to increase the hybridization rate even further. In either case, the use of such modified oligonucleotides in diagnostic methods leads to more rapid identification of biological analytes, which in turn leads to, for example, to more effective treatments for disease conditions caused by or indicated by such analytes.

As described in more detail below, Applicant has found that probes displaying a preferential affinity to RNA targets may be used to specifically detect RNA over DNA of the same sequence (except that U is substituted for T in the RNA sequence). Such methods have application in, for example, the specific detection of RNA viruses in cells containing DNA versions of the viral genome, or in specific detection of levels of RNA transcription in cells.

Probes may also be devised which contain both target-complementary sequences as well as additional target, non-complementary sequences. These target, non-complementary sequences may have other functions. For example, the sequences may be complementary to another oligonucleotide or target nucleic acid; or they may have functional properties, such as promoter sequences and restriction sites. Thus, a probe may have more than one function, only one of which is to be detected as an indication of the presence of a target.

Additionally, probes may be designed to have at least one nucleic acid strand which has at least two separate target-complementary sequences that can hybridize to a target nucleic acid. An example of such probes is described by Hogan et al., U.S. Patent Nos.

5,424,413 and 5,451,503, which enjoy common ownership with the present application and are expressly incorporated by reference herein. The probes disclosed by Hogan *et al.* further include at least two distinct arm regions that do not hybridize with the target, but possess complementary regions that are capable of hybridizing with one another. These arm regions can be designed to require the presence of target in order for their complementary sequences to hybridize under suitable hybridization conditions. Accordingly, target-complementary sequences of the probe must hybridize to the target before complementary arm regions can hybridize to each other. The resulting structure is termed a branched nucleic acid.

Other probes may be designed which are unable to specifically bind the target. To be useful in detecting target, these probes must be able to hybridize with another nucleic acid that can bind with the target, either directly or indirectly. In one such arrangement, a nucleic acid can be structured to contain at least a first and second, nonoverlapping nucleotide base sequence regions, where the first nucleotide region is complementary to a nucleotide base sequence of the target and the second nucleotide region is complementary to a nucleotide base sequence of the probe. In this arrangement, the second nucleotide region of the nucleic acid would be unavailable for binding with the probe until the target has hybridized with the first nucleotide region of the nucleic acid. Binding of the nucleic acid and target would alter the configuration of the nucleic acid, thus permitting the second nucleotide region of the nucleic acid to bind with the probe. See Figure 2. Of course, this arrangement could be modified so that indirect binding between the nucleic acid and target, accomplished with one or more intervening or coupling nucleic acids, would render the second nucleotide region of the nucleic acid available for binding with the probe.

Modified Nucleic Acid Amplification Oligonucleotides

In still other aspects, the present invention includes methods for employing modified oligonucleotide primers, promoter-primers, and/or splice templates for nucleic acid amplification and compositions comprising such oligonucleotides, wherein the

oligonucleotides contain at least one cluster of modified bases which cause an increased rate of hybridization.

Primer-employing amplification methods include the polymerase chain reaction method (PCR) and its variations, as described by Mullis, *et al.*, (see U.S. Patent Nos. 4,683,195, 4,683,202, and 4,800,159, European Patent Application Nos. 86302298.4, 86302299.2, and 87300203.4, and 155 *Methods in Enzymology*, 335-350 (1987)). The PCR methodology is by now a matter of common knowledge to those skilled in the art.

PCR has been coupled to RNA transcription by incorporating a promoter sequence into one of the primers used in the PCR reaction and then, after amplification by the PCR method, using the double-stranded DNA as a template for the transcription of single-stranded RNA. (See, *e.g.*, Murakawa, *et al.*, *DNA*, 7:287-295 (1988)).

Other amplification methods use multiple cycles of RNA-directed DNA synthesis and transcription to amplify DNA or RNA targets. See, *e.g.*, Burg, *et al.*, U.S. Patent No. 5,437,990; 89/1050; Gingeras, *et al.*, WO 88/10315; Davey and Malek, EPO Publication No. 0 329 822; Malek, *et al.*, WO91/02818, Kacian and Fultz, U.S. Patent No. 5,480,783; McDonough, *et al.*, WO 94/03472; and Kacian, *et al.*, WO 93/22461 (the latter three of these publications enjoy common ownership with the present application and are incorporated by reference herein). Urdea, *et al.*, WO91/10746, describe a method that achieves signal amplification using a T7 promoter sequence.

Each of these methods makes use of one or more oligonucleotide primers or splice templates able to hybridize to or near a given nucleotide sequence of interest. After hybridization of the primer, the target-complementary nucleic acid strand is enzymatically synthesized, either by extension of the 3' end of the primer or by transcription, using a promoter-primer or a splice template. In some amplification methods, such as PCR, rounds of primer extension by a nucleic acid polymerizing enzyme is alternated with thermal denaturation of complementary nucleic acid strands. Other methods, such as those of Kacian & Fultz, *supra*, McDonough, *et al.*, *supra*, and Kacian, *et al.*, *supra*, are isothermal transcription-based amplification methods.

In each amplification method, however, side reactions caused by hybridization of the primer to non-target sequences can reduce the sensitivity of the target-specific reaction. These competing "mismatches" may be reduced by raising the temperature of the reaction. However, raising the temperature may also lower the amount of target-specific primer binding as well.

Thus, according to this aspect of the invention, primers having high target affinity, and comprising modified nucleotides in the target binding region, may be used in nucleic acid amplification methods to more sensitively detect and amplify small amounts of a target nucleic acid sequence, by virtue of the increased temperature, and thus the increased rate of hybridization to target molecules, while reducing the degree of competing side-reactions (cross-reactivity) due to non-specific primer binding. Preferred oligonucleotides contain at least one cluster of modified bases, but less than all nucleotides are modified in preferred oligonucleotides.

In another preferred embodiment, modified oligonucleotide primers are used in a nucleic acid amplification reaction in which a target nucleic acid is RNA. See, e.g., Kacian and Fultz, supra. The target may be the initially present nucleic acid in the sample, or may be an intermediate in the nucleic acid amplification reaction. In this embodiment, the use of preferred 2'-modified primers, such as oligonucleotides containing 2'-O-methyl nucleotides, permits their use at a higher hybridization temperature due to the relatively higher T_m conferred to the hybrid, as compared to the deoxyoligonucleotide of the same sequence. Also, due to the preference of such 2'-modified oligonucleotides for RNA over DNA, competition for primer molecules by non-target DNA sequences in a test sample may also be reduced. Further, in applications wherein specific RNA sequences are sought to be detected amid a population of DNA molecules having the same (assuming U and T to be equivalent) nucleic acid sequence, the use of modified oligonucleotide primers having kinetic and equilibrium preferences for RNA permits the specific amplification of RNA over DNA in a sample.

Sample Processing

In accord with the present invention, modified oligonucleotides having increased target-specific hybridization kinetics and binding affinities as compared to their unmodified analogues may be used in a variety of hybridization assay sample processing methodologies.

By sample processing is meant methods allowing or enhancing the discrimination of analyte and non-analyte nucleic acids. Such methods may involve, for example, the direct or indirect immobilization of nucleic acids or oligonucleotides from the liquid phase in a heterogeneous assay. Some such methods may involve two or more hybridization events resulting in such immobilization.

For example, Ranki, et al., U.S. Patent Nos. 4,486,539 and 4,563,419, discuss a one-step nucleic acid "sandwich" hybridization method involving the use of a solid-phase bound nucleic acid having a target complementary sequence and a labeled nucleic acid probe, complementary to a separate portion of the target nucleic acid. Stabinsky, U.S. Patent No. 4,751,177, discusses methods involving the use of a "mediator" polynucleotide which reportedly overcomes sensitivity problems in the Ranki method associated with leakage of the immobilized oligonucleotide from the solid support.

Other methods may employ an immobilized oligonucleotide, for example an oligonucleotide containing a homopolymeric tract, such as poly T, or a simple short repeating sequence, and two or more coupling oligonucleotides, one of which is able to hybridize with the immobilized oligonucleotide and a different one of which is able to specifically hybridize with target. Each of the coupling oligonucleotides is able to bind at least one other coupling oligonucleotide. If a coupling oligonucleotide does not contain a sequence complementary to the target or immobilized oligonucleotide, it will be able to hybridize with at least two other coupling oligonucleotides simultaneously. The solid support may be comprised of materials including nitrocellulose, a polymeric substance, such as polyacrylamide or dextran, metallic substances or controlled pore glass. The support may be in forms such as a sheet, membrane or a particle. Additionally, the solid support may have a magnetic charge to facilitate

recovering sample and/or washing away unbound nucleic acids or other sample components.

Joining of the immobilized oligonucleotide to the solid support may be accomplished by any method that will continue to bind the immobilized oligonucleotide throughout the assay steps. Additionally, it is important that when the solid support is to be used in an assay, it be essentially incapable, under assay conditions, of the non-specific binding or adsorption of non-target oligonucleotides or nucleic acids.

Common immobilization methods include binding the nucleic acid or oligonucleotide to nitrocellulose, derivatized cellulose or nylon and similar materials. The latter two of these materials form covalent interactions with the immobilized oligonucleotide, while the former binds the oligo through hydrophobic interactions. When using these materials it is important to use a "blocking" solution, such as those containing a protein, such as bovine serum albumin (BSA), or "carrier" nucleic acid, such as salmon sperm DNA, to occupy remaining available binding sites on the solid support before use in the assay.

Other immobilization methods may include the use of a linker arm, for example, N-hydroxysuccinamide (NHS) and its derivatives, to join the oligonucleotide to the solid support. Common solid supports in such methods are, without limitation, silica, polyacrylamide derivatives and metallic substances. In such a method, one end of the linker may contain a reactive group (such as an amide group) which forms a covalent bond with the solid support, while the other end of the linker contains another reactive group which can bond with the oligonucleotide to be immobilized. In a particularly preferred embodiment, the oligonucleotide will form a bond with the linker at its 3' end. The linker is preferably substantially a straight-chain hydrocarbon which positions the immobilized oligonucleotide at some distance from the surface of the solid support. However, non-covalent linkages, such as chelation or antigen-antibody complexes, may be used to join the oligonucleotide to the solid support.

A desirable embodiment of the latter assay system contains two coupling oligonucleotides: (i) a first coupling oligonucleotide containing a nucleotide sequence substantially complementary to the immobilized oligonucleotide, for example, with a poly A

nucleotide sequence complementary to a poly T sequence on the immobilized oligo, and (ii) a second coupling oligonucleotide containing a nucleotide sequence substantially complementary to the target nucleic acid, a detectably labeled probe, or both. In a preferred embodiment, the second coupling oligonucleotide contains a nucleotide sequence substantially complementary to the target nucleic acid. Moreover, each coupling oligonucleotide in the preferred embodiment contains another nucleotide sequence which enables the first and second coupling oligonucleotides to hybridize to each other under assay conditions. However, one or more additional coupling oligonucleotides may be introduced into the system, such that the first and second coupling oligonucleotides are indirectly bound to each other by means of these additional, intervening coupling oligonucleotides. The additional coupling oligonucleotides would be substantially unable to hybridize with any of the target nucleic acid, the detectably labeled oligonucleotide probe, or the immobilized oligonucleotide under assay conditions.

Yet another assay system having practical advantages in ease and rapidity of use may comprise an immobilized oligonucleotide having a portion complementary to a capturing oligonucleotide. The capturing oligonucleotide (capture probe) will contain a base sequence permitting hybridization to the target. The capturing oligonucleotide will also have a label attached within or near the target-binding nucleotide sequence region, such as a substituted or unsubstituted acridinium ester, which may be used in a homogeneous or semi-homogeneous assay system to specifically detect hybrid nucleic acids without detecting single-stranded nucleic acids, such as the capture probe itself. Such a system favored by Applicant is the HPATM, which is discussed and incorporated by reference above. In the HPATM format, the label contained on any capture probe which has not hybridized to its target will be hydrolyzed with the addition of base, while target:capture probe hybrid would protect the label associated therewith from hydrolysis.

An advantage to this latter assay system is that only one target-specific hybridization event (labeled capture probe:target) need occur for target detection, rather than two such events (capture probe:target and labeled probe:target) in the other sample processing

procedures described herein. Fewer oligonucleotides in the assay would tend to make the assay faster and simpler to optimize, since the overall rate at which labeled target is captured is limited by the slowest hybridizing probe. Additionally, while the portion of the target complementary to the capturing oligonucleotide in these other assay systems does not have to be as specific as the target's probe binding region, this base sequence must be rare enough to avoid significant saturation of the capture probe with non-target nucleic acids. Thus, this preference for two separate and specific target sequences may place constraints on finding an appropriate target to which such assays are to be directed. By contrast, only one such target sequence need be found in the latter assay, since the same nucleotide sequence functions simultaneously to immobilize and detect the target nucleic acid.

Regardless of the approach used, a necessary element of any assay is a method of detection of the desired target. A number of options known to those of skill in the art are possible. One such option is the direct use of a labeled nucleic acid probe. Such a probe would have a nucleotide sequence region which is specifically hybridizable with and substantially complementary to the target nucleic acid of interest. Upon hybridization to the target and immobilization of the target:probe hybrid, unbound probe can be washed away or inactivated and the remaining label hybrid-associated detected and/or measured.

Another option combines the elements of detection and nucleic acid amplification. In such a system, the target nucleic acid is immobilized as described, for example, and without limitation, in the assay procedures described above. One or more amplification oligonucleotides (see, e.g., Kacian, et al., WO93/22461), such as a primer, promoter-primer, or splice template, able to hybridize with a specific region of the target nucleic acid may be contacted with the immobilized target nucleic acid under nucleic acid amplification conditions, e.g., in the presence of one or more nucleic acid polymerases and ribo- and/or deoxyribonucleotide triphosphates.

The resulting polynucleotide strand (amplicon) can be made directly available for specific hybridization and detection with a labeled hybridization assay probe or for further amplification by hybridizing the polynucleotide strand with one or more additional

amplification oligonucleotides under nucleic acid amplification conditions. If the latter option is chosen, the amplification reaction can be continued until the desired level of amplification is achieved, then the resulting amplicons, which may comprise copies of at least a portion of the immobilized target nucleic acid, polynucleotides complementary to at least a portion of the immobilized nucleic acid, or both, can be detected using one or more labeled oligonucleotide probes. If the amplification reaction is to take place while the target is immobilized, it is important that the portion of the target molecule to be used as a template for the amplicons not contain the nucleotide sequence region necessary for immobilization of the target nucleic acid. Although amplicons of either or both senses can be detected with the labeled probes, in a preferred embodiment only amplicons of the opposite sense to, i.e., complementary to, the immobilized target are detected.

A heterogeneous target capture method such as this is particularly advantageous since crude clinical samples can contain substances which inhibit or interfere with the amplification reaction. Thus, the ability to separate the target nucleic acid from such interfering substances can permit or enhance the sensitivity of nucleic acid amplification.

This solid-phase associated amplification scheme can be used in myriad assay systems, including those described above. Applicant currently prefers an assay system employing one or more coupling oligonucleotides, as described above, which are able to indirectly link the target nucleic acid to the solid support. It is also preferred that the complementary nucleotide sequence regions of the support-coupled oligonucleotide, and the capturing oligonucleotide designed to hybridize to it, be at least partially homopolymeric or contain simple repeating nucleotide sequences, so as to promote rapid hybridization. Additionally, or alternatively, these regions of either or both the immobilized oligonucleotide and the capturing oligonucleotide may be modified in a manner consistent with the disclosure of this specification to increase the rate of hybridization between these oligonucleotides. In such a system, the target capture oligonucleotide and the target nucleic acids are preferably allowed to hybridize in solution before hybridizing to the immobilized oligonucleotide. In the currently preferred embodiment, the immobilized target is washed, the amplification

oligonucleotide (or oligonucleotides) is contacted with the immobilized target under nucleic acid amplification conditions, and following amplification the labeled amplicon-directed probe is added and detected.

Applicant prefers to use the transcription-based amplification method described in Kacian & Fultz, supra, previously incorporated by reference. In accord with this method, a promoter-primer having a 3' region complementary to a portion of the target and a 5' region and a primer having the same nucleotide sequence as a portion of the target are contacted with a target RNA molecule. The primer and promoter-primer define the boundaries of the target region to be amplified, including both the sense present on the target molecule and its complement, and thus the length and sequence of the amplicon. In this preferred embodiment, the amplification oligonucleotides and immobilized target RNA are contacted in the presence of effective amounts of Moloney murine leukemia virus-derived reverse transcriptase and T7 RNA polymerase, both ribonucleotide and deoxyribonucleotide triphosphates, and necessary salts and cofactors at 42°C. Under these conditions, nucleic acid amplification occurs, resulting predominantly in production of RNA amplicons of a sense opposite to that of the target nucleic acid. These amplicons are then detected, e.g., by using an acridinium ester-labeled hybridization assay probe of the same sense as the target-nucleic acid, in the hybridization protection assay disclosed in Arnold, supra, previously incorporated by reference.

In this preferred embodiment, Applicant prefers that the 3' terminus of the immobilized oligonucleotide, target capture oligonucleotide and coupling oligonucleotide(s) be "capped" or blocked to prevent or inhibit their use as templates for nucleic acid polymerase activity. Capping may involve addition of 3'-deoxyribonucleotides (such as cordycepin), 3', 2'-dideoxynucleotide residues, non-nucleotide linkers, such as disclosed in Arnold, et al., supra, alkane-diol modifications, or non-complementary nucleotide residues at the 3' terminus.

Although Applicant currently prefers to contact the primers with the target following target immobilization, there may be hybridization kinetic advantages to combining the target nucleic acid and at least one primer complementary thereto at the same time that the target

capture oligonucleotide is added. Applicant believes that it is advantageous to conduct the target hybridization in solution prior to immobilization of the target, as hybridization can take place in solution more rapidly than when one nucleic acid is immobilized.

5 Likewise, while Applicant prefers to form and detect amplicons of the opposite sense to the target, there is no reason why one could not form and detect amplicons of either or both senses. Additionally, when amplifying target nucleic acids contained in crude clinical samples, it appears important to conduct a wash step prior to the amplifying step to prevent enzyme inhibition and/or nucleic acid degradation due to substances present in the sample.

10 It will be clear to the skilled person that this methodology is amenable, either as described or with obvious modifications, to various other amplification schemes including the polymerase chain reaction.

Modified Oligonucleotides in Sample Processing

15 Modified oligonucleotides able to hybridize to complementary targets with increased kinetics may be used in sample processing methods which employ nucleic acid hybridization, including the target capture methods described above. In light of the present disclosure it will be apparent that such partly or wholly modified oligonucleotides may be employed as hybridization assay probes or amplification oligonucleotides in these systems. Additionally,
20 wholly or partially modified oligonucleotides having increased target-directed hybridization kinetics may be used as immobilized oligonucleotides, target capture oligonucleotides, and/or one or more coupling oligonucleotides in heterogenous assays employing nucleic acid hybridization. For example, such modifications may be used to reduce the overall assay time or to allow the hybridization steps of the assay to occur at a single temperature. The
25 advantages of reduced time and ease of assay operation in a clinical setting gained thereby would be clear to those skilled in the art.

Additionally, oligonucleotides may be modified to have hybridization kinetics and/or equilibrium preferences for a specific type of nucleic acid, such as RNA or DNA. As

disclosed above, for example, 2'-O-methyl oligonucleotides preferentially hybridize with RNA over DNA. Thus, target capture oligonucleotides containing 2'-O-methyl nucleotides may be used to specifically capture RNA target nucleic acids, such as mRNA or rRNA, under hybridization conditions not promoting hybridization of the oligonucleotide to the genomic versions thereof. Likewise, 2'-O-methyl-modified amplification oligonucleotides and/or labeled probes can be designed, thereby targeting RNA over DNA for amplification and/or detection, as described above.

Modified Helper Oligonucleotides

Helper oligonucleotides are described in the Hogan, *supra*. Helper oligonucleotides are generally unlabeled and used in conjunction with labeled hybridization assay probes to increase the labeled probe's T_m and hybridization rate by "opening up" target nucleotide sequence regions which may be involved in secondary structure, thus making these regions available for hybridization with the labeled probe.

In light of the present disclosure, those of skill in the art will easily recognize that using modified helper oligonucleotides which will hybridize with the target nucleic acid at an increased rate over their unmodified counterparts can lead to even greater hybridization rates of the labeled probe to their target. Thus, methods and compositions for detecting oligonucleotides employing such modified helper oligonucleotides are intended to be encompassed within the scope of this invention. Preferred helper oligonucleotides have modifications which give them a greater avidity towards RNA than DNA. In a preferred embodiment, such modifications include a cluster of at least about 4 2'-O-methyl nucleotides. In a particularly preferred embodiment, such modifications would include a cluster of about 8 2'-O-methyl nucleotides.

Diagnostic Kits

The methods described herein also clearly suggest diagnostic kits specially formulated for use in such methods. These kits will contain one or more oligonucleotides to be used in a diagnostic nucleic acid hybridization assay. At least one of these oligonucleotides will contain a cluster of at least about 4 modified nucleotides designed to hybridize to a target nucleic acid region at an increased rate over an otherwise identical oligonucleotide.

Such diagnostic kits may include, without limitation, one or any combination of the probe, amplification, helper and sample processing oligonucleotides described herein.

In a preferred embodiment of the present invention, the kit contains at least one labeled oligonucleotide probe having a region containing one or more clusters of at least about 4 contiguous 2'-modified nucleotide residues. In a more preferred embodiment, the region contains one or more clusters of about 8 2'-modified nucleotides.

Applicant currently prefers using an acridinium ester derivative as a non-radioactive label and the addition of a methoxy group as a 2' modification. In a particularly preferred embodiment, at least one of the modified oligonucleotides will comprise one or more clusters of at least about 4 2'-O-methyl nucleotides. Even more preferred is at least one oligonucleotide containing one or more clusters of about 8 2'-O methyl nucleotides.

Kits containing one or more of the modified oligonucleotides disclosed herein could be sold for use in any diagnostic hybridization assay method, or related amplification method, of the present invention. In such an assay, at least one of the modified oligonucleotides contained in the kit would function as a probe able to hybridize to a target nucleic acid. If the modified probe is contacted with a sample containing the target nucleic acid, the probe will exhibit improved hybridization properties over an unmodified probe having an identical base sequence. For instance, the hybridization binding affinity between the target and the probe will be greater than the hybridization binding affinity between the target and an unmodified form of the probe, when subjected to the same hybridization assay conditions. Additionally, the hybridization rate between the target and the probe will be greater than the

hybridization rate between the target and an unmodified form of the probe, when subjected to the same hybridization assay conditions.

To further improve the hybridization properties of the probe, one or more conjugate molecules may bound to the probe, preferably in a region containing a cluster of at least about 4 modified nucleotides. It is also expected that the kit would be packaged with instructions for using one or more modified oligonucleotides in a diagnostic hybridization assay of the present invention.

OBJECTS

It is therefore an object of the present invention to provide methods for increasing the both the avidity of binding and the hybridization rate between a diagnostic nucleic acid probe and its target nucleic acid by utilizing probe molecules having one or more modified nucleotides, preferably a cluster of about 4 or more, and more preferably about 8, modified nucleotides. In preferred embodiments, the modifications comprise 2' modifications to the ribofuranosyl ring. In most preferred embodiments the modifications comprise a 2'-O-methyl substitution.

It is also an object to provide methods for increasing the rate of hybridization of a single-stranded oligonucleotide to a target nucleic acid through the incorporation of a plurality of modified nucleotides into the oligonucleotide. An increased rate of hybridization accomplished in this manner would occur over and above the increase in hybridization kinetics accomplished by raising the temperature, salt concentration and/or the concentration of the nucleic acid reactants.

It is another object of the invention to provide diagnostic methods for selectively targeting RNA over DNA through the use of oligonucleotides modified to have an increased target binding efficiency and to hybridize to RNA at an enhanced rate over DNA. In a preferred embodiment, such oligonucleotides comprise a 2'-O-methyl modification to the ribofuranosyl ring.

It is an additional object of the invention to provide sample processing methods which

employ an immobilized oligonucleotide to directly or indirectly capture target nucleic acids. In preferred embodiments, such methods employ one or more oligonucleotides which can specifically hybridize to the target nucleic acid, permitting its detection and immobilization. In a preferred embodiment, a single labeled oligonucleotide is responsible for both capture
5 and detection of the target. In a particularly preferred embodiment, a coupling or bridging nucleic acid is bound to both the immobilized oligonucleotide and the oligonucleotide responsible for capture and detection of the target. Additional coupling nucleic acids are possible. Some or all of the oligonucleotides used in sample processing methods may contain modifications which accelerate the rate of target specific hybridization.

10 It is yet another object of the invention to provide target specific oligonucleotides of between about 10 and about 100 bases, preferably between about 10 and about 15 bases, and more preferably between about 12 and about 15 bases, which preferably contain at least one cluster of at least about 4 nucleotides, more preferably about 8 nucleotides, modified to increase their target-specific binding efficiency while simultaneously increasing their
15 discrimination between target and non-target nucleotide sequences as compared to longer unmodified oligonucleotides designed to hybridize to the same site.

It is a further object of the present invention to provide kits including one or more oligonucleotides containing modified nucleotides which function to increase the rate of hybridization between the oligonucleotide and a target nucleic acid. Kits of the present
20 invention could include any combination of probe, amplification, helper and sample processing oligonucleotides. In a preferred embodiment, the modified oligonucleotides of these kits would contain at least one cluster of about 4 2'-O-methyl modifications to the ribofuranosyl ring. Kits containing these modified oligonucleotides may be supplied for use in both diagnostic hybridization assays and amplification assays. Such kits may further
25 include written instructions directing practitioners in the use of the modified oligonucleotides in either or both diagnostic hybridization assays or amplification assays.

The diagnostic methods of the present invention are, therefore, specially adapted to exploit the hybridization properties of modified oligonucleotides having increased binding

affinity. These methods may be used for the detection or quantification of any target nucleic acid. In a preferred embodiment, the target nucleic acid is RNA. The methods may employ "chimeric" oligonucleotides composed of regions of unmodified oligodeoxy- or oligoribonucleotides combined with regions of modified oligonucleotides or may utilize wholly modified oligonucleotides. Preferably, the oligonucleotides are not wholly modified. The regions may be designed simply to promote rapid hybridization of probe to target, or may have other functions. For example, a chimeric oligonucleotide may be designed to bind both to RNA and to DNA. In such a case, the RNA-binding portion of the oligonucleotide may contain a plurality of modified nucleotides to preferentially bind the RNA target. Alternatively, the region of modified residues may be designed to be directed towards a target present in low abundance in order to increase the hybridization rate.

Given the present disclosure, it will be understood that certain embodiments of the methods and compositions, including the kits, of the present invention may employ oligonucleotides having more than one type of modification affecting the hybridization properties of the resulting oligonucleotide, i.e., T_m and hybridization kinetics. Such multiple modifications may act in a cooperative fashion to further increase the hybridization rate or to increase the specificity of the resulting oligonucleotide for a given type of nucleic acid target, such as RNA. Furthermore, chimeric oligonucleotides may have or consist of regions of differently modified oligonucleotides containing either 2'- modified nucleotides or nucleotides having other modifications or both.

The objects and aspects of the invention specifically described herein are not intended as an exhaustive listing of the objects or aspects of the methods and compositions of the present invention which would be apparent to those skilled in the art in light of the present disclosure. Nor should the preceding description or the Examples which follow be construed as limiting the invention to the embodiments specifically disclosed therein.

Examples

Unless otherwise indicated, in all the following examples oligodeoxyribonucleotides, oligoribonucleotides, and modified oligonucleotides were synthesized by use of standard phosphoroamidite chemistry, various methods of which are well known in the art. See e.g., Carruthers, et al., 154 Methods in Enzymology, 287 (1987), which is hereby incorporated by reference as part of this disclosure. Unless otherwise stated herein, modified nucleotides were 2' O-methyl-nucleotides, which were used in the synthesis as their phosphoramidite analogs. Applicant prepared the oligonucleotides using an Expedite 8909 DNA Synthesizer (PerSeptive Biosystems, Framingham, MA).

Also, unless otherwise indicated, oligonucleotides indicated as labeled contained an acridinium phenyl ester. Acridinium phenyl ester compounds are derivatives of acridine possessing a quaternary nitrogen center and derivatized at the 9 position to yield a phenyl ester moiety. However, leaving groups other than phenyl moieties are well known in the art. Acridinium esters have the property of reacting with hydrogen peroxide to form a transient dioxetane ring involving the C-9 carbon of the acridinium ring, followed by the formation of an excited acridone. The radiative relaxation of the excited acridone results in the production of light. The synthesis of acridinium esters, as well as a general description of their use as chemiluminescent labeling reagents, is described in Weeks, et al., Acridinium Esters as High Specific Activity Labels in Immunoassays, Clin. Chem., 29:1474-1478 (1984), which is incorporated by reference herein.

In these Examples, the acridinium esters were attached, using standard chemical techniques, to a non-nucleotide monomeric unit having a primary amine "linker arm" joined to the acridinium ester moiety, which is inserted between contiguous sequences of nucleotides during the chemical synthesis of the oligonucleotides, or placed at a terminal position of the oligonucleotide. See, Arnold, et al., Non-Nucleotide Linking Reagents for Nucleotide Probes, EPO Publication No. EPO 313219, which enjoys common ownership with the present invention, and is now incorporated by reference herein. However, it will be understood that

the preference of 2'-modified oligonucleotides for RNA targets and the effect of the modified oligonucleotides on the rate of hybridization to DNA targets are not determined by the presence or specific nature of a label. Thus, those of skill in the art will recognize that oligonucleotides used in the methods of the present invention may be labeled with a variety of labels, or they may be unlabelled when, for example, they are used as amplification primers, helper oligonucleotides or in a capture assay.

Acridinium ester derivatives may be joined to the linker arm: hybridization probe conjugate using techniques well known in the art. Preferably, Applicant uses the methods described in Nelson, *et al.*, Detection of Acridinium Esters by Chemiluminescence in Non-Isotopic Probe Techniques (Academic Press 1992), and Arnold, *et al.*, Non-Nucleotide Linking Reagents for Nucleotide Probes, EPO Publication No. EPO 313219, previously incorporated by reference herein.

Further, unless expressly indicated otherwise, all target nucleic acids were RNA.

It will nevertheless be clear to those of skill in the art, in light of the present disclosure, that other labels may be used in the methods and compositions of the present invention without departing from the spirit of the invention disclosed herein.

Example 1: Effect of 2' Modifications on the T_m of Probe:Target Hybrids

Oligonucleotide probes of identical sequence containing varying amounts of 2'-O-methyl nucleotides were each individually hybridized to perfectly complementary synthetic RNA targets of the same length. The probe sequence had SEQ ID NO:1 and the target sequence had SEQ ID NO:2. The nucleotide base sequences of these oligonucleotides were as follows:

SEQ ID NO:1	5'-GCTCGTTGCG GGA CTT(AE)AACC CAACAT-3'
SEQ ID NO:2	5'-ATGTTGGGTT AAGTCCCGCA ACGAGC-3'

The probes, as illustrated below, were synthesized to contain no 2'-O-methyl nucleotides (Probe A), all 2'-O-methyl nucleotides (Probe B), or a combination of deoxy- and 2'-O-methyl nucleotides (Probes C, D and E). Probe C contained four contiguous deoxyribonucleotides positioned directly adjacent to each side of the linker attachment site and 2'-O-methyl ribonucleotides at all other bases; Probe D contained four contiguous 2'-O-methyl nucleotides positioned directly adjacent to each side of the linker attachment site and deoxyribonucleotides at all other bases, and Probe E contained eight contiguous 2'-O-methyl nucleotides positioned directly adjacent to each side of the linker attachment site and deoxyribonucleotides at all other bases. The T_m of each hybrid was determined using both a chemiluminescent and an optical method.

Chemiluminescent Method

Using the chemiluminescent method, approximately 1 pmol of the RNA target and 0.1 pmol of each oligonucleotide probe, labeled as described above with "standard" acridinium ester (4-(2-succinimidylloxycarbonyl ethyl) phenyl-10-methylacridinium 9-carboxylate fluorosulfonate) were allowed to hybridize at 60°C for 60 minutes in 30 µl of lithium succinate buffer (1.5 mM EDTA (ethylenediaminetetraacetic acid), 1.5 mM EGTA (ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid), 310 mM lithium lauryl sulfate, 0.1 M lithium succinate (pH 5.2)). The resulting solution was then diluted to 500 µl with lithium succinate buffer, and 50 µl aliquots were incubated at various temperatures for 7 minutes. Each sample was then cooled on ice for an additional 7 minutes. The acridinium ester coupled to unhybridized probe molecules was hydrolyzed by adding 150 µl of a solution containing 190 mM $\text{Na}_2\text{B}_4\text{O}_7$ (pH 7.6), 7% (v/v) TRITON® X-100 (polyoxyethylene p-t-octyl phenol) and 0.02% (w/v) gelatin, and the samples were heated at 60°C for 10 minutes. The remaining (hybrid associated) chemiluminescence of each sample was determined in a LEADER® 50 luminometer (MGM Instruments; Hamden, Ct.) by the automatic injection of a solution containing 0.1% v/v H_2O_2 in 0.001 M HNO_3 followed 0.5 - 2 seconds later by an injection of 200 µl of 1N NaOH. The resulting light emission was integrated over a 2 second

interval.

Optical Method

Using the optical method, an identical set of oligonucleotide probes were synthesized having a linker arm but were not labeled with an acridinium ester. Four micrograms of each oligonucleotide probe were allowed to hybridize to 4 μ g of the complementary RNA target for 60 minutes at 60°C in 30 μ l of a hybridization buffer containing 200 mM lithium hydroxide, 3 mM EDTA, 3 mM EGTA, 17% w/v lithium lauryl sulfate, and 190 mM succinic acid (pH 5.2). Following hybridization, 600 μ l of the hybridization buffer was added, the sample split into two, and the melting behavior of each sample portion examined on a Beckman DU640 spectrophotometer equipped with a Micro T_m analysis accessory. The temperature was varied 1°C per minute for temperatures that were more than 10°C either lower or higher than the T_m and 0.5°C per minute at intervals of 0.2°C for all other temperatures. Changes in hypochromaticity were monitored and recorded as a function of temperature. Results are shown in Table I below.

Table I

<u>Probe</u>	<u>Number of modified nucleotides</u>	<u>T_m (chemiluminescent)</u>	<u>T_m (optical)</u>	<u>ΔT_m</u>
A	0	90	72.4	0
B	26	90	91.2	22 ; 18.8
C	18	83	87.9	15; 15.5
D	8	72	76.4	4; 4
E	16	nd	84.2	11.6

nd = not done

As shown in Table 1, the T_m data generated using the chemiluminescent and optical methods agreed well with each other. The somewhat lower T_m values observed with the chemiluminescent method can be attributed to the lower nucleic acid concentrations used in the chemiluminescent method versus the optical method. The data show that replacement of
5 all of the deoxyribonucleotide residues of Probe A with 2'-O-methyl nucleotides (Probe B) resulted in probe:RNA target hybrids having a T_m increased by about 20.4°C. Probes C, D and E exhibited T_m increases of 15°C, 4°C, and 11.6°C, respectively. By calculating the effect on T_m for each substitution of a 2'-O-methyl nucleotide, these data reveal that the T_m of the 2'-O-methyl oligonucleotide:RNA target hybrid increases about 0.8°C for every such
10 replacement. This effect is approximately linear over the number of substitutions tested.

Example 2: Effect of 2'-Modified Nucleotides on T_m of Probe:rRNA Hybrids

Three sets of oligonucleotide probes of different length and sequence were
15 synthesized, and each set contained two oligonucleotides of identical base sequence. Probe F was 17 bases in length and included an acridinium ester label joined at a site located between a thymine base and an adenine base. Probe G was 18 bases in length and likewise included an acridinium ester label joined at a site located between a thymine base and an adenine base.
Probe H was 20 bases in length and included an acridinium ester label joined at a site
20 located between a thymine base and a guanine base.

Each set of probes contained one oligonucleotide consisting entirely of deoxyribonucleotides and another oligonucleotide containing only 2'-O-methyl nucleotides. Each probe was then hybridized to the corresponding ribosomal RNA, and the T_m of the resulting hybrids determined by the chemiluminescent method described above. The results
25 are shown in Table 2 below.

Table 2

<u>Probe</u>	<u>Length (bases)</u>	<u>T_m (deoxy)</u>	<u>T_m (2'-O-methyl)</u>	<u>Δ T_m</u>	<u>Δ T_m per modified nucleotide</u>
F	17	63	81	18	1.05
G	18	66	78	12	0.66
H	20	62	75	13	0.65

The data confirm the results of Example 1, showing that replacement of a deoxyribonucleotide with a 2'-O-methyl nucleotide increases the T_m of the resulting probe:RNA target hybrid. Additionally, when calculated as the average of the three probes' increase in T_m per modified nucleotide, the contribution of each modified nucleotide was an increase of 0.8°C per modified nucleotide.

Example 3: Effect of 2'-Modified Nucleotides on T_m of Probe:DNA Hybrids

In this Example, the effect of 2'-modification on probe:DNA targets was tested. Probe I, which contained varying amounts of 2'-O-methyl nucleotides, was hybridized to an exactly complementary DNA target of the same length and the melting behavior of the resultant hybrids examined by the chemiluminescent method described above. Probe I was 29 bases in length and included an acridinium ester label joined at a site located between a thymine base and a guanine base.

Probe I was designed to consist of: (i) all deoxyribonucleotides; (ii) all 2'-O-methyl nucleotides; and (iii) all 2'-O-methyl nucleotides except for four deoxyribonucleotides, which were positioned immediately on each side of the label attachment site. Results of the T_m determination are shown in Table 3 below.

Table 3

<u>Probe</u>	<u>Number of 2'-O-methyl nucleotides</u>	<u>T_m</u>	<u>Δ T_m per modified nucleotide</u>
I	0	69	0
I	29	77	0.28
I	21	75	0.29

As the data shows, replacement of deoxyribonucleotides with 2'-O-methyl nucleotides in Probes J and K caused the T_m of the labeled probe:DNA target to increase approximately 0.3°C per 2'-O-methyl residue.

A similar test was done using three sets of different oligonucleotides. Each set contained two oligonucleotides, one of the oligonucleotides containing deoxyribonucleotides and the other containing 100% 2'-O-methyl nucleotides, having identical base sequences. Probe J was 16 bases in length and included an acridinium ester label joined at a site located between a thymine base and an adenine base. Probe K was 18 bases in length and likewise included an acridinium ester label joined at a site located between a thymine base and an adenine base. Probe L was 29 bases in length and included an acridinium ester label joined at a site located between a thymine base and a guanine base.

In each case the synthetic DNA targets were completely complementary to the probes. The results are shown in Table 4 below.

Table 4

<u>Probe</u>	<u>Number of 2'-O-methyl nucleotides</u>	<u>T_m (optical method)</u>	<u>Δ T_m per modified nucleotide</u>
J	0	75.3	0
J	26	74.5	-0.03

K	0	71.6	0
K	19	67.0	-0.24
L	0	74	0
L	29	78.2	0.14

The data contained in Tables 3 and 4 demonstrates that the T_m of DNA targets is increased to a significantly lesser degree than RNA targets when 2'-O-methyl substitutions are introduced into the probes.

Example 4: Analysis of the Stabilities of Different Types of Nucleic Acid Hybrids

To compare the relative stabilities of hybrids containing various combinations of DNA, RNA, and 2'-O-methyl nucleotide strands, acridinium ester-labeled oligonucleotide probes of SEQ ID NO:1 (see Example 1 above) were hybridized to synthetic targets having a perfectly complementary base sequence. The probes and target sequences contained 100% ribonucleotides (RNA), 100% deoxyribonucleotides (DNA) or 100% 2'-O-methyl nucleotides in the combinations indicated in Table 5. The melting characteristics of each tested hybrid, as determined either using the chemiluminescent or the optical method, is shown in Table 5 below. More than one data point in the table indicates an independent, duplicate experiment.

Table 5

<u>Probe</u>	<u>Target</u>	<u>T_m (chemiluminescent)</u>	<u>T_m (optical)</u>
DNA	RNA	68, 67	73.3, 73.6, 73, 72.4
RNA	RNA	81	nd
2'-O-methyl nucleotides	RNA	87, 90	91.2
2'-O-methyl nucleotides	2'-O-methyl nucleotides	91	nd
DNA	DNA	nd	75.5, 75.7, 75.1, 75.4
2'-O-methyl nucleotides	DNA	nd	74.3, 74.8

nd = no data

Thus, this experiment indicates that the stability of labeled probe:target hybrids follows the order: 2'-O-methyl/2'-O-methyl \geq 2'-O-methyl/RNA > RNA/RNA > DNA/DNA > 2'-O-methyl/DNA > DNA/RNA

Example 5: Ability of Enhanced Stability of 2'-Modified Oligo:RNA Hybrids to Allow Specific RNA Targeting

As indicated in Example 4, 2'-O-methyl:RNA hybrids are considerably more stable than 2'-O-methyl:DNA hybrids. To illustrate that this difference in stability can be exploited in a diagnostic assay to specifically detect RNA molecules over DNA molecules having an identical sequence (but with uracil in the RNA replacing thymine in the DNA), the following experiment was done.

An acridinium ester-labeled oligonucleotide probe of SEQ ID NO: 1 (see Example 1 above) and composed of 100% 2'-O-methyl nucleotides was allowed to hybridize to a completely complementary synthetic RNA or DNA target. Other than the fact that the oligonucleotide was labeled, hybridization and measurement of T_m were otherwise as described in Example 1 under the heading Optical Method. The results are shown in Table 5

above and are further illustrated in Figure 3.

As indicated in Figure 3, at 81.6°C the 2'-O-methyl oligonucleotide forms a detectable hybrid with the RNA target, but not with the DNA target. By contrast, Table 5 demonstrates that when a labeled DNA oligonucleotide of the same sequence is hybridized with the identical RNA or DNA targets, the resulting hybrids have substantially similar melting characteristics.

Thus, according to the aspect of the present invention demonstrated here, it is possible to specifically detect RNA targets in preference to DNA targets under easily determined hybridization conditions. Such methods may be used, as a non-exclusive example, to specifically detect various RNA species, such as mRNA, tRNA, or rRNA, without interference from the identical sequence existing in the genomic DNA of the organism being assayed. Such methods may be useful for applications including monitoring the rate of expression of a particular gene product. Other uses exploiting this ability of the 2'-modified oligonucleotides will be apparent to those of skill in the art.

Example 6: Effect of 2'-Modified Nucleotides on the Hybridization Kinetics of Oligonucleotides

The effect of 2'-modified nucleotides on hybridization kinetics was illustrated using four different methods. The probe molecules used in this example were labeled with standard acridinium ester as described previously.

a) In the first approach, 2 fmol of an acridinium ester-labeled probe having SEQ ID NO: 1 (see Example 1 above) was hybridized to varying amounts of a completely complementary RNA target for a constant period of time, followed by differential hydrolysis and detection of the label. The hybridization was performed essentially as described in Example 1, under the heading Chemiluminescent Method, with the following differences. Varying amounts of RNA target were allowed to hybridize with the labeled probe at 60°C for 45 minutes. Figure 4 shows the results of this experiment, wherein the probe was either a DNA oligonucleotide

(open boxes) or consisted wholly of 2'-O-methyl nucleotides (closed diamond); these results are also tabulated in Table 6 below. The degree of hybridization is expressed in Relative Light Units (rlu), which is a measure of the number of photons emitted by the acridinium ester label.

Table 6

<u>Amount of Target (fmol)</u>	<u>rlu (DNA probe)</u>	<u>rlu (2'-O-methyl)</u>
1	855	1,739
5	3,394	8,009
10	5,476	14,217
50	13,810	27,959
100	18,798	34,381
200	19,199	31,318

The results indicate that the hybridization rate, as a function of target concentration, is significantly increased when the probe contains 2'-O-methyl nucleotides rather than unmodified nucleotides. This holds true throughout the range of target concentrations studied. For comparison purposes, the initial slopes of these data are used to estimate the relative hybridization rates of deoxy- (slope = 1.0) and 2'-O-methyl (slope= 2.5) oligonucleotide probes.

b) In a second approach, a constant amount (2 fmol) of the same target used in a) above was hybridized to varying amounts of the perfectly complementary probe for a fixed amount of time. Figure 5 shows the results of this experiment, wherein the probe was either a DNA oligonucleotide (open boxes) or consisted wholly of 2'-O-methyl nucleotides (closed boxes). The hybridization and detection steps were the same as described in a) above, except that the

hybridization reaction was carried out for 30 minutes rather than 45 minutes. The data are tabulated in Table 7 below.

Table 7

<u>Amount of Probe (fmoles)</u>	<u>rlu (DNA probe)</u>	<u>rlu (2'-O-methyl)</u>
0.2	99	346
0.5	309	966
1	690	1,973
2	1,206	4,356
5	3,227	9,184
10	5,801	14,615
20	7,289	21,515
50	13,080	33,236
100	15,223	34,930

Again, the results in this example indicate that the hybridization rate, which is a function of probe concentration, is significantly increased when the probe contains 2'-O-methyl nucleotides rather than unmodified nucleotides. The slopes of these plots are similar to those of Figure 4, indicating that regardless of whether probe concentration or target concentration is varied, the difference in hybridization kinetics between 2'-O-methyl/DNA and DNA/DNA interactions remains the same. In this experiment, the initial slope of the reaction containing the DNA probe was 1.0 and of the reaction containing the 2'-O-methyl probe was 3.1.

c) As a third illustration of the ability of 2'-modified oligonucleotides to increase the rate

of hybridization, fixed amounts of either modified or unmodified probe (1 fmol) and target (100 amol) were allowed to hybridize for varying amounts of time. The hybridization and detection protocols were otherwise the same as in b). Figure 6 shows the results, wherein the probe was either a DNA oligonucleotide (open boxes) or consisted wholly of 2'-O-methyl nucleotides (closed diamonds). The data were as follows in Table 8 below:

Table 8

<u>Time (minutes)</u>	<u>rlu (DNA probe)</u>	<u>rlu (2'-O-methyl)</u>
0	118	613
1	124	627
2	200	732
6.5	294	978
10.5	500	1331

Again, the relative rates of hybridization can be determined from the initial slopes of the curves (deoxy = 1.0; 2'-O-methyl = 2.2). In this experiment, the initial slope of the reaction containing the DNA oligonucleotide was 1.0, and the initial slope of the reaction containing the 2'-O-methyl oligonucleotide probe was 2.2-fold.

d) The fourth method used to demonstrate the differences between the hybridization kinetics of 2'-modified and unmodified probes was a C_{0t} analysis. Acridinium ester-labeled probes of SEQ ID NO:1 (see Example 1 above) were used. Either a fixed amount of probe and varied amounts of target ("probe excess") or a fixed amount of target and varying amounts of probe ("target excess") were allowed to hybridize at 60°C for varying amounts of time. The fixed amount of either probe or target was 0.25 fmol and the variable amount of

either probe or target included amounts in the range from 0.25 to 50 fmol. Hybridization was otherwise as indicated in Example 1, under the heading Chemiluminescent Method.

Differential hydrolysis of the unhybridized acridinium ester and detection of the hybridized probe was accomplished by adding 150 μ l of 190 mM $\text{Na}_2\text{B}_4\text{O}_7$ (pH 7.6), 7% (v/v) TRITON[®] X-100 and 0.02% (w/v) gelatin to the sample, and heating the mixture to 60°C for 10 minutes. Chemiluminescence was read in a luminometer, as described above. Percent maximal hybridization was defined as the ratio of the observed rlu value divided by the maximal rlu value observed when saturating amounts of probe or target were used in the hybridization reaction.

In the C_0t plot shown in Figure 7, the quantity $\ln(1-H)$ is plotted against the concentration of target times the hybridization time. The value H is defined as the percent hybridization of the probe at a particular target concentration after a particular time.

In this plot, the relative rates of hybridization of DNA and 2'-O-methyl oligonucleotide probes is given by the inverse of the relative ratios of C_0t at 50% hybridization ($\ln(1-0.5)$; deoxy = 1.0 and 2'-O-methyl = 2.2).

A summary of the relative hybridization rates of an acridinium ester-labeled probe consisting entirely of deoxy- or 2'-O-methyl ribonucleotides determined by these four methods is summarized in Table 9. The data resulting from these experiments indicate that at 60°C an oligonucleotide consisting entirely of 2'-O-methyl nucleotides hybridizes 2.3-fold faster than the corresponding deoxyribonucleotide probe.

Table 9

<u>Method</u>	<u>Probe (fmol)</u>	<u>Target (fmol)</u>	<u>Hybridization Time</u>	<u>Relative Rate (2'-O-methyl/ deoxy-)</u>
c	0.05	1	vary	2.3
c	10	0.5	vary	2.3
c	0.1	1	vary	2.0
c	1	0.2	vary	1.6
c	3	0.3	vary	2.9
c	10	1	vary	1.8
b	vary	1	30	2.6
b	vary	2	45	3.1
a	2	vary	45	2.5
d	0.25	vary	44	2.2

Example 7: Kinetic Analysis of Hybridization of 2'-Modified Oligonucleotides to Additional Targets

To extend these hybridization rate comparisons to other probe and target sequences, Probe H of Example 2 above was synthesized entirely of either deoxy- or 2'-O-methyl nucleotides and hybridized to rRNA in the presence of helper probes. A C_0t analysis was performed, as in Example 6(d). The results are shown in Table 10 below.

Table 10

<u>Probe</u>	<u>C₀t</u>	<u>Relative Rates</u>
deoxy	0.3×10^7	1.00
2'-O-methyl	8×10^7	3.75

In this Example, the probe consisting entirely of 2'-O-methyl nucleotides hybridized 3.75-fold faster than the deoxyribonucleotide probe of identical sequence. Thus, enhanced hybridization by acridinium ester-labeled probes containing 2'-O-methyl nucleotides does not appear to be limited to any particular probe or target sequence.

Example 8: Effect of Increased Temperature on Hybridization Rate of 2'-Modified Oligonucleotides

As mentioned above, nucleic acid hybridization kinetics are accelerated by an increase in temperature. However, the advantages of this acceleration can be offset by the destabilizing effects of increased temperature on duplex formation, especially in diagnostic assays and nucleic acid amplification procedures employing relatively short oligonucleotides (between about 10 and about 50 bases in length). As shown below, the increased duplex stability provided by oligonucleotides modified as presently described can minimize this destabilizing effect, allowing the hybridization rate to be further increased by conducting the hybridization at a higher temperature than would otherwise be possible. Thus, a cooperative effect on hybridization kinetics is provided by the modified oligonucleotides and the higher hybridization temperature.

An acridinium ester-labeled oligonucleotide probe of SEQ ID NO:1 (see Example 1 above) consisting entirely of 2'-O-methyl nucleotides was allowed to hybridize to a perfectly complementary RNA target of the same length as described above. The hybridization and C₀t protocol were as described in Example 6(d), except that hybridization temperatures were

either 60°C or 70°C. As shown by the data in Table 11 below, raising the temperature of hybridization of the 2'-O-methyl oligonucleotide to its target from 60°C or 70°C caused the hybridization kinetics to be accelerated 1.5 fold.

Table 11

<u>Hybridization Temperature</u>	<u>C₀t_{0.5}</u>	<u>Relative Hybridization Rates</u>
60°C	1.7×10^{-5}	1
70°C	1.1×10^{-5}	1.5

Example 9: Effect of Increasing Salt Concentration on Hybridization Kinetics of 2'-Modified Oligonucleotides

Hybridization kinetics are also accelerated by increases in salt concentration. The following example illustrates the effect of various concentrations of salt, e.g., LiCl, on the hybridization kinetics of 2'-O-methyl nucleotides. An acridinium ester-labeled probe of SEQ ID NO:1 (see Example 1 above), consisting entirely of 2'-O-methyl nucleotides, was allowed to hybridize, and a C₀t analysis conducted, as described in Example 6(d) above, at 80°C to an exactly complementary RNA target of the same length. Hybridization was performed at two different concentrations of LiCl. As shown in Table 12 below, increasing the salt concentration from 0.5 to 1.0 M LiCl enhanced the hybridization kinetics 2.9 fold.

Table 12

<u>LiCl Concentration</u>	<u>C₀t_{0.5}</u>	<u>Relative Rates</u>
0.5 M	0.72×10^3	1
1.0 M	0.25×10^3	2.9

These results demonstrate that a two-fold increase in the salt concentration in a hybridization reaction leads to a 2.9-fold increase in the hybridization kinetics of modified oligonucleotides.

Example 10: Combined Effect on Hybridization Kinetics of Increasing Salt Concentration and Temperature

To demonstrate the effect of simultaneously increasing the hybridization temperature and salt concentration on the hybridization kinetics of 2'-modified oligonucleotides, the following reactions were performed. An acridinium ester-labeled DNA oligonucleotide and an acridinium ester-labeled oligonucleotide having 100% 2'-O-methyl nucleotides, both of SEQ ID NO:1 (see Example 1 above), were each separately allowed to hybridize to an exactly complementary RNA target molecule in a C₀t analysis. Hybridization conditions were as described in Example 6(d). Hybridization temperature and salt concentrations were as indicated in Table 13 below. The results were as follows:

Table 13

<u>Probe</u>	<u>Hybridization Temperature</u>	<u>LiCl Concentration</u>	<u>C₀t_{0.5}</u>	<u>Relative Rates</u>
DNA	60°C	0.5 M	1.9×10^3	1
2'-O-methyl	80°C	1.0 M	0.22×10^3	8.6

As the data indicate, at a hybridization temperature of 80°C and in 1.0M LiCl, the 2'-O-methyl oligonucleotide hybridized to its target at an 8.6-fold faster rate than did the corresponding DNA oligonucleotide at a temperature of 60°C and salt concentration of 0.5M LiCl.

Example 11: Comparison of Hybridization Rates of RNA, DNA and 2'-Modified Oligonucleotides Hybridizing to Complementary DNA and RNA Targets

The relative hybridization rates of labeled RNA, DNA and 2'-O-methyl-containing oligonucleotides to completely complementary DNA and RNA targets were individually determined. Rate determination was performed as disclosed in either Example 6(c) or 6(d) above. The labeled oligonucleotides had identical base sequences of SEQ ID NO:1 (see Example 1 above). The results are summarized in Table 14 below:

Table 14

<u>Probe</u>	<u>Target</u>	<u>Initial Slope</u>	<u>C₀t₁</u>	<u>T_m</u>	<u>Relative Rates</u>
DNA	DNA	.0031, .0048	---	75.4	4.4
RNA	DNA	---	---	74.5	---
2'-O Me	DNA	.0014, .0014	---	74.6	1.6
DNA	RNA	.0009	---	73.3	1
RNA	RNA	---	1.1 x 10 ⁻⁵ , 1.4 x 10 ⁻⁵	81	2.8
2'-O Me	RNA	.004	.71 x 10 ⁻⁵ , .89 x 10 ⁻⁵	91.2	4.4

Experiments represented in Rows 1 through 4 were done as disclosed in Example 6(c) using 3 fmol of labeled probe and 0.3 fmol of target. The experiments represented in rows 5 and 6 were performed using the method described in Example 6(d). Where more than one result is indicated, each value corresponds to a different individual experiment.

The results of Table 14 demonstrate that substitution of deoxyribonucleotide residues

with either 2'-OH residues (RNA) or 2'-O-methyl residues enhances the affinity, as well as the hybridization kinetics, of the probe to an RNA target. In contrast, substitution of deoxyribonucleotide residues with 2'-O-methyl residues does not enhance the affinity or the hybridization kinetics of the probe to a DNA target.

5 The results presented in Table 14 reveal that substitution of deoxyribonucleotide residues with 2'-O-methyl residues enhances the affinity and hybridization kinetics of a probe for an RNA target, but not a DNA target. However, these experiments do not eliminate the possibility that the acridinium ester label and/or the linker, by which it is attached to the probe, may be responsible for these hybridization rate characteristics. To show that the
10 acridinium ester label and/or linker do not appreciably affect hybridization rates, the following experiment was performed.

An RNA probe of SEQ ID NO:1 labeled with acridinium ester (see Example 1 above) and containing a non-nucleotide linker by which the label was attached to the probe was allowed to hybridize to an exactly complementary target which consisted entirely of either 2'-
15 O-methyl or deoxyribonucleotides. Hybridization and C_{ot} analysis was done as Example 6(d). The results are expressed in Table 15 below.

Table 15

<u>Labeled Probe</u>	<u>Target</u>	<u>C_{ot}</u>	<u>Relative Rates</u>
RNA	DNA	6.2×10^{-5}	1
RNA	2'-O-methyl	2.3×10^{-5}	2.7

20 These data reveal that substitution of deoxyribonucleotides with 2'-O-methyl residues enhances the hybridization kinetics of an oligonucleotide lacking an acridinium ester and/or linker. Thus, the increased hybridization rate observed for 2'-O-methyl modified probes is not due, to the presence of a label or linker arm, but is an intrinsic property of the 2'-O-methyl-modified oligonucleotide.

Example 12: Comparative Effect of Helper Probes on the Hybridization of Probes from DNA Probe Mixes and 2'-Modified Probe Mixes to an rRNA Target

As disclosed in Hogan, supra, the hybridization of some probes to nucleic acids, especially those having a significant amount of secondary structure, is facilitated through the use of additional probes, termed "helper" probes. An example, though not the exclusive example, of a nucleic acid species having a high degree of secondary structure is ribosomal RNA (rRNA). Helper probes can help disrupt secondary structure which may mask the target region. The helper probe is usually targeted to a sequence region near, but preferably not overlapping with, the probe target sequence. In practice, helper probes are generally not labeled, and are usually used in a large molar excess. The effect of using helper probes on labeled probe hybridization is usually expressed as an increased T_m and hybridization rate for the labeled probe:target hybrid.

The following experiments were performed to examine whether 2'-modified oligonucleotide probes have the same requirements for helper probes as do DNA probes when directed to given target regions containing a large amount of secondary structure. Two probe mixes were made. Each probe mix contained Probes F, G and H of Example 2 above. The oligonucleotides of one probe mix were made up of 100% 2'-O-methyl nucleotides, and the oligonucleotides of the other probe mix were composed entirely of deoxyribonucleotides. Where indicated, probe mixes included unlabeled DNA helper probes a, b, c, d, e and f having lengths of 33, 36, 41, 31, 29 and 40 bases, respectively.

Each helper probe was directed to rRNA base sequences close to the target site of one of the labeled probes. The degree of hybridization was measured using the hybridization protection assay (HPATM), as described above. The results are reported in relative light units (rlu).

As shown in Table 16 below, in the absence of helper probes, DNA probe mixes hybridized poorly to rRNA. In contrast, when probe mixes employing 2'-O-methyl probes of identical sequence to the DNA probe mixes were hybridized to rRNA in the absence of helper probes, much higher levels of hybridization were observed. Additionally, when helper probes

were used with both probe mixes, significantly greater hybridization of the probes to their target occurred with the 2'-modified oligonucleotide probe mixes. Because the hybridization of a DNA probe to an rRNA depends strongly on the presence of helper probes, while the hybridization of an identical 2'-O-methyl probe does not, 2'-O-methyl probes can efficiently hybridize to highly structured RNA molecules, such as ribosomal RNA, under conditions where DNA probes cannot.

Table 16

<u>Probe</u>	<u>Helpers</u>	<u>rRNA Concentration</u> <u>(amol)</u>	<u>rlus</u>
DNA	no	100	14
DNA	yes	100	3185
2'-O-methyl	no	100	3116
2'-O-methyl	yes	100	4332
DNA	no	1,000	68
DNA	yes	1,000	24,912
2'-O-methyl	no	1,000	19,934
2'-O-methyl	yes	1,000	33,584
DNA	no	10,000	730
DNA	yes	10,000	204,876
2'-O-methyl	no	10,000	148,386
2'-O-methyl	yes	10,000	256,940

The data in Table 16 further indicate that helper probes are not needed to facilitate probe binding when 2'-modified oligonucleotides are used. However, even greater sensitivity than seen before can be achieved in assays employing both helper probes and 2'-modified

probes.

Example 13: Comparative Effect of Helper Probes on the Hybridization of a Single DNA Probe and a Single 2'-Modified Probe to an rRNA Target

5

The results of the experiments described in Example 12 were generated using three separate labeled probes, either with or without the presence of the six helper oligonucleotides identified in Example 12. In this example, Probes F and H of Example 2, and consisting
10 entirely of deoxyribo- or 2'-O-methyl nucleotides, were allowed to hybridize to target rRNA in the presence or absence of the indicated helper probes. Helper probes a, b, c and d of Example 12 above were used. Table 17 shows the hybridization characteristics of DNA and 2'-O-methyl oligonucleotides of Probe A. Table 18 shows the hybridization characteristics of
15 DNA and 2'-O-methyl oligonucleotides of Probe H. Each labeled probe was tested in the presence or absence of the various helper probes and helper probe combinations indicated.

Table 17

<u>Probe</u>	<u>Target Concentration</u> <u>(amol)</u>	<u>Helper Probes</u>	<u>rlus</u>
deoxy	500	None	153
deoxy	500	a	5,600
deoxy	500	b	761
deoxy	500	a and b	9,363
2'-O-methyl	500	None	14,537
2'-O-methyl	500	a	16,556
2'-O-methyl	500	b	15,586
2'-O-methyl	500	a and b	16,868
deoxy	1,000	None	1,060
deoxy	1,000	a	14,782
deoxy	1,000	b	316
deoxy	1,000	a and b	26,877
2'-O-methyl	1,000	None	28,874
2'-O-methyl	1,000	a	23,201
2'-O-methyl	1,000	b	16,269
2'-O-methyl	1,000	a and b	44,510

Table 18

<u>Probe</u>	<u>Helper</u>	<u>RLUs</u>
deoxy	c	870
deoxy	d	9,176
deoxy	c and d	47,745
2'-O-methyl	c	88,292
2'-O-methyl	c	69,943
2'-O-methyl	c and d	98,663

Tables 17 and 18 demonstrate that the labeled DNA probes required helper oligonucleotides in order to effectively hybridize to their targets under the assay conditions. Additionally, Table 18 shows that the 2'-modified oligonucleotides hybridized to their targets to a greater degree in the absence of helper probes than the DNA oligonucleotides hybridized to the same target in the presence of the added helpers. Finally, 2'-O-methyl oligonucleotides exhibited even greater hybridization properties in the presence of helper oligonucleotides.

Example 14: Comparative Effect of Temperature on the Hybridization Properties of DNA Probes and 2'-Modified Probes with an rRNA Target in the Presence and Absence of Helper Probes

The data presented in Example 13 indicates that 2'-O-methyl oligonucleotides hybridize to a detectable extent to RNA targets, even highly folded structures like rRNA, in the absence of helper probes. Nevertheless, helper probes can accelerate the hybridization of 2'-O-methyl oligonucleotides to highly structured RNA. To examine the effect of helper probes more closely, deoxy- and 2'-O-methyl oligonucleotide probes were hybridized to rRNA at different temperatures in the presence or absence of helper probes. Table 19 represents studies performed using acridinium ester-labeled probes having a nucleotide

sequence of Probe F of Example 2 above and helper probes c and d of Example 12 above. Table 20 represents studies performed using acridinium ester-labeled probes having a nucleotide sequence of SEQ ID NO:1 (see Example 1 above) and helper probes g and h having 41 and 32 bases, respectively.

Table 19

Probe	Helpers	Temperature	C.L.	Relative Rate
deoxy	Yes	60°C	26.4×10^{-5}	1.3
2'-O-methyl	No	60°C	35×10^{-5}	1
2'-O-methyl	Yes	60°C	8.15×10^{-5}	4.3
2'-O-methyl	No	75°C	12.9×10^{-5}	2.7
2'-O-methyl	Yes	75°C	4.12×10^{-5}	8.5

Table 20

Probe	Helpers	Temperature	C.L.	Relative Rate
2'-O-methyl	No	60°C	7.46×10^{-5}	1
2'-O-methyl	Yes	60°C	1.77×10^{-5}	4.2

These experiments demonstrate that at 60° and 75°C, helper probes enhanced the hybridization rates of the 2'-O-methyl probes to their targets 3.1-4.3 fold.

Example 15: Effect of 2'-Modified Nucleotides on the Hydrolysis Properties of Acridinium Ester-Labeled Probes

As a further demonstration of the effect of modified oligonucleotides on the performance characteristics of diagnostic probe molecules, a number of additional experiments

were performed. These experiments were based on the Applicant's preferred detection method employing the HPATM detection assay. In accordance with one HPATM format, a chemiluminescent acridinium ester is attached to a probe and the probe is hybridized to an analyte. Following hybridization, chemiluminescence associated with unhybridized probe is
5 selectively destroyed by brief hydrolysis in borate buffer. Since probe:analyte molecules are not destroyed in this process, the remaining chemiluminescence of hybridized probe is a direct measure of the analyte present. In this application, those acridinium ester-labeled probes which hydrolyze faster when unhybridized than when in a probe-analyte hybrid complex are preferred. Hydrolysis of probe and hybrid is pseudo first order and can be
10 characterized by the value $t_{1/2}$, which is the time, measured in minutes, required to hydrolyze 50% of the acridinium ester attached to either probe or hybrid. Thus, probes which exhibit a large differential hydrolysis (DH) ratio ($t_{1/2}(\text{hybrid})/t_{1/2}(\text{probe})$) are highly desirable.

To examine the effect of modified oligonucleotides on the hydrolysis properties of acridinium ester-labeled probes, four sets of probes were constructed, each set having a
15 distinct nucleotide base sequence and each member of a set having identical nucleotide base sequences. Each set of probes contained one probe consisting entirely of unmodified nucleotides and another probe consisting entirely of 2'-O-methyl nucleotides. The probes used were Probes A and B of Example 1 above and Probes F, G and H of Example 2 above. DH ratios of each probe to an exactly complementary RNA target were determined as
20 described above, for example, in Example 1. As summarized in Table 21 below, unhybridized probes containing deoxy- or 2'-O-methyl nucleotides hydrolyzed at very similar rates.

Table 21

	<u>Probe</u>	<u>Ribonucleotides</u>	<u>t_{1/2} (Probe)</u>	<u>t_{1/2} (Hybrid)</u>	<u>DH</u>
A	A	deoxy	.81	49.1	60.3
	B	2'-O-methyl	.63	77.2	123.5
B	F	deoxy	.36	20.79	7.74
	F	2'-O-methyl	.89	75.25	4.55
C	H	deoxy	.69	17.26	25
	H	2'-O-methyl	.76	44.7	58.8
D	G	deoxy	.62	25.67	41.4
	G	2'-O-methyl	.81	23.55	29.7

10 In contrast, the modified probe:target hybrid-associated label for three of the sequences was approximately 2-fold more resistant to hydrolysis than in the otherwise identical unmodified probe:target hybrid. In one case, in which the probe contained an ATAT sequence surrounding the acridinium ester linker, the DH ratio was decreased 1.4-fold for the modified probe:target hybrid-associated label.

15 Example 16: Effect of Position of 2'-Modified Nucleotides on the Hydrolysis Properties of Acridinium Ester-Labeled Probe

20 To examine whether modified nucleotides must be close to the site of label attachment to enhance the DH behavior of acridinium ester, the acridinium ester-labeled probes of SEQ ID NO:1, containing clusters of 2'-O-methyl nucleotides at different positions relative to the acridinium ester linker site, were hybridized to a complementary RNA target. Those nucleotides containing 2'-O-methyl substitutions are indicated below by underlining:

Probe M: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'

Probe N: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'

Probe O: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'

Probe P: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'

5

As shown in Table 22 below, the measurements reveal that 4 contiguous 2'-O-methyl nucleotides on either side of the acridinium ester linker site are sufficient to enhance the DH behavior of an acridinium ester-labeled probe as much as a probe consisting entirely of 2'-O-methyl nucleotides. More than one data point in the table indicates independent, duplicate experiments.

10

Table 22

<u>Probe</u>	<u>t_{1/2} (Probe)</u>	<u>t_{1/2} (Hybrid)</u>	<u>DH</u>
M	.82, .8	48.7, 43.2	59.7, 54
N	.76, .6	90, 77.6	118.3, 129
O	.74	49.8	67.3
P	.44	81.4	185

15

Example 17: Effect of Temperature on the Hydrolysis Properties of 2'-Modified Acridinium Ester-Labeled Probes

20

As mentioned above, because of their higher thermal stability, the modified oligonucleotides of the present invention are able to hybridize to a target nucleic acid at a higher temperature than unmodified oligonucleotides. At such higher temperatures, the hybridization rate, as well as the rate of other reactions, would be expected to increase. Among such other reactions is the rate of hydrolysis of acridinium ester labels. Because Applicant's preferred detection method employs the Hybridization Protection Assay (HPA™),

25

described and incorporated by reference above, the following experiment was performed to determine whether benefits to the diagnostic assay conferred by an increase in hybridization rate would be offset by a decrease in the DH ratios of hybrid-associated and unassociated acridinium ester labels at this higher temperature.

5 An acridinium ester-labeled probe consisting entirely of 2'-O-methyl nucleotides was allowed to hybridize to a complementary RNA target at 60°, 70° and 80°C. Hybridization conditions were as otherwise as described previously.

As summarized in Table 23 below, at 70°C and 80°C acridinium ester-labeled probes containing 2'-O-methyl nucleotides exhibited DH ratios comparable to acridinium ester-
10 labeled probes containing deoxyribonucleotides at 60°C. Thus, elevated temperature may be used in diagnostic assays employing the methods and compositions of the present invention without a detectable decrease in assay sensitivity due to degradation of the label.

Table 23

15

<u>Temp (°C)</u>	<u>Probe</u>	<u>t_{1/2} (Probe)</u>	<u>t_{1/2} (Hybrid)</u>	<u>DH</u>
60	deoxy	.82	48.7	59.7
60	2'-O-methyl	.76	90	118
70	2'-O-methyl	.42	25.7	61.3
80	2'-O-methyl	.25	10.1	40.8

20

Example 18: Effect of 2'-Modified Nucleotides on the Hydrolysis Properties
of Various Acridinium Ester-Labeled Probes

25 The foregoing experiments were conducted using standard acridinium ester as the detectable chemiluminescent label. To examine whether the differential hydrolysis behavior of labels other than standard acridinium ester is enhanced by T_m-enhancing modified

nucleotides, probes of SEQ ID NO: 1 and containing either deoxy- or 2'-O-methyl nucleotides were labeled in exactly the same manner and position (see Example 1 above) with standard acridinium ester, o-diBr acridinium ester, 2-Me acridinium ester, naphthyl-acridinium ester, o-F acridinium ester, 2,7-diisopropylacridinium ester, or mixture of 1- and 3-Me acridinium ester, and their DH behavior examined. See Figure 1 for examples of acridinium esters. As summarized in Table 24 below, the use of modified nucleotide probes resulted in an increase in the DH ratio for all the acridinium ester derivatives tested by 1.1-6 fold.

Table 24

<u>Label</u>	<u>Probe</u>	<u>t_{1/2} (Probe)</u>	<u>t_{1/2} (Hybrid)</u>	<u>DH</u>
Standard Acridinium ester	deoxy	.81	49.1	60.3
Standard Acridinium ester	2'-O-methyl	.63	77.2	123.5
o-diBr-acridinium ester	deoxy	.94	23.44	24.94
o-diBr-acridinium ester	2'-O-methyl	1.01	66.65	66
2-Me-acridinium ester	deoxy	.84	73.6	87.62
2-Me-acridinium ester	2'-O-methyl	.78	101.8	130.5
Naphthyl acridinium ester	deoxy	.72	14.45	20.07
Naphthyl acridinium ester	2'-O-methyl	.57	53.4	93.68
o-F acridinium ester	deoxy	.93	53.3	57.3

o-F acridinium ester	2'-O-methyl	1.03	78.75	76.46
2,7-diisopropyl acridinium ester	deoxy	1.23	38.55	31.3
2,7-diisopropyl acridinium ester	2'-O-methyl	0.8	43.90	54.9
Mixture of 1- and 3-Me acridinium ester	deoxy	.97	12.6	129.8
Mixture of 1- and 3-Me acridinium ester	2'-O-methyl	1.24	149.5	120.6

Example 19: Relationship Between the Hybridization Kinetics and the Number of 2'-Modified Nucleotides Contained in a Probe Sequence

To examine the relationship between hybridization kinetics and the number of 2'-O-methyl nucleotides within a probe sequence, acridinium ester-labeled probes of SEQ ID NO:1 (see Example 1 above) were synthesized. These synthesized probes contained increasing amounts of 2'-O-methyl residues (underlined bases indicate the presence of 2'-O-methyl residues) on both sides of the AE linker:

Probe Q: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'

Probe R: 5'-GCTCGTTGCG GGACTT(AE)AAACC CAACAT-3'

Probe S: 5'-GCTCGTTGCG GGACTT(AE)AAACC CAACAT-3'

Probe T: 5'-GCTCGTTGCG GGACTT(AE)AAACC CAACAT-3'

Probe U: 5'-GCTCGTTGCG GGACTT(AE)AAAACAACAT-3'

Probe V: 5'-GCTCGTTGCG GGACTT(AE)AAAAACAACAT-3'

The results are summarized in Table 25 below.

Table 25

<u>2'-O-methyl Residues</u>	<u>C_t (Exp 1)</u>	<u>C_t (Exp 2)</u>	<u>C_t (Exp 3)</u>	<u>Relative Rate</u>
0	1.87×10^{-5}	2.2×10^{-5}	---	1
2	---	---	1.13×10^{-5}	1.8
4	1.28×10^{-5}	---	0.93×10^{-5}	1.8
8	---	0.93×10^{-5}	0.86×10^{-5}	2.3
16	---	---	0.96×10^{-5}	2.1
25	0.91×10^{-5}	1×10^{-5}	0.84×10^{-5}	2.2

As summarized above, as few as 8 2'-O-methyl nucleotides (Probe T) -- 4 on each side of the acridinium ester linker site -- were sufficient to accelerate the hybridization rate of an acridinium ester probe to the same level of a probe consisting almost entirely of 2'-O-methyl nucleotides (Probe V). In contrast, the T_m of a probe containing four 2'-O-methyl nucleotides on each side of the acridinium ester linker site is lower than the T_m of a probe:target hybrid in which the probe contains additional 2'-O-methyl nucleotides. Thus, according to the present invention it is possible to optimize, or "tune", the performance of an acridinium ester labeled probe with respect to its hybridization rate, differential hydrolysis, and melting properties. For example, an acridinium ester-labeled probe containing four 2'-O-methyl nucleotides on either side of the acridinium ester linker site will have its hybridization rate and differential hydrolysis properties maximally optimized, while a hybrid containing this probe will exhibit only a small increase in its melting temperature.

As substantially contiguous 2'-O-methyl nucleotides are added to replace deoxyribonucleotides in the labeled probe, the hybridization rate and differential hydrolysis

properties of the probe:target hybrid will remain substantially constant while its T_m will continue to increase. The ability to increase, incrementally, the influence of a probe on the T_m of a probe:target hybrid allows one to adjust the specificity of a probe so as not to cross-react with closely related sequences, as shown in Table 25 above.

5

Example 20: Effect of Propyne-Modifications on the T_m of Probe:Target Hybrids

In order to illustrate the general usefulness of the compositions and methods of the present invention in the diagnostic application of nucleic acid hybridization technology, oligonucleotides were constructed having a modification other than a 2'-modification to the ribofuranosyl moiety, but which also caused an increase in the binding affinity of a probe for its target. In this example, oligonucleotides were synthesized containing two nucleotides modified at the nitrogenous base. Specifically, *N*-diisobutylaminomethylidene-5-(1-propynyl)-2'-deoxycytidine; (a cytidine analog) and 5-(1-propynyl)-2'-deoxyuridine) (a thymidine analog). These nucleotide analogs are commercially available, for example, from Glen Research in Sterling, VA.

10

15

20

25

As a first consideration, probes having 22 bases and an acridinium-ester attached at a site located between a thymine base and a guanine base in Probes W and Y and between two thymine bases in Probe X, but containing varying numbers of propyne-modified nucleotides, were hybridized to target rRNA in the presence of helper probes to examine the effect of the modification on the T_m of acridinium ester hybrids. Probe W contained no propyne modifications. Probe X contained two propyne modifications, one directly adjacent to each side of the label attachment site. Probe Y contained 11 propyne modifications, including four contiguous modifications directly adjacent and 5' to the label attachment site and seven modifications located at bases spaced 3, 4, 6, 9-11 and 14 bases away from and 3' to the label attachment site.

Hybridization and T_m determinations were performed as described above using detection of acridinium ester-labeled hybrids. As summarized below in Table 26, these data

indicate that the T_m of the oligonucleotide, when hybridized to an RNA target, increased an average of 1°C for every replacement of a pyrimidine with a propyne-substituted pyrimidine.

Table 26

Probe	Propyne Residues	T_m (chemiluminescent)	$\Delta T/\text{Propyne}$
W	0	71	---
X	2	72	0.5
Y	11	82	1.0

Example 21: Effect of Propyne Modifications on the Hybridization Kinetics of Oligonucleotides

To examine the effect of propyne groups on the hybridization kinetics of oligonucleotides, the rate of hybridization of the propyne-labeled probes of Example 20 to RNA were examined by C_{ot} analysis, as described in Example 6. As summarized below in Table 27, the probe containing two propyne groups (Probe W) hybridized at the same rate as the probe containing no propyne groups (Probe X), while the probe containing 11 propyne groups (Probe Y) hybridized 1.9-fold faster.

Table 27

Probe	C_{ot}	Relative Rate
W	0.75×10^{-3}	1
X	0.81×10^{-3}	0.93
Y	0.39×10^{-3}	1.9

5 These data support the generality of the present invention by demonstrating that modifications to oligonucleotides which result in an increased T_m also cause the rate of hybridization of the modified oligonucleotide to its target to increase compared to an unmodified oligonucleotide of the same base sequence. Moreover, this example also demonstrates that such modifications may occur in the nitrogenous base moiety as well as the sugar moiety. Those of skill in the art will recognize that such modifications may also occur in the internucleotide linkage as well.

10 Although the foregoing disclosure describes the preferred embodiments of the present invention, Applicant should not be limited thereto. Those of skill in the art to which this invention applies will comprehend additional embodiments in light of this disclosure. Moreover, additional embodiments are within the claims which conclude this specification and their equivalents.

SEQUENCE LISTING

- 5 (I) GENERAL INFORMATION
- (i) APPLICANTS: Becker, Michael M.
Majlessi, Mehrdad
- 10 (ii) TITLE OF THE INVENTION: Methods for Detecting and Amplifying
Nucleic Acid Sequences Using Modified
Oligonucleotides Having Increased Target
Specific T_m
- 15 (iii) NUMBER OF SEQUENCES: 2
- (iv) CORRESPONDENCE ADDRESS:
- 20 (A) ADDRESSEE: Gen-Probe Incorporated
(B) STREET: 9880 Campus Point Drive
(C) CITY: San Diego
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 92121
- 25 (v) COMPUTER READABLE FORM:
- 30 (A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: MS-DOS Version 6.22
(D) SOFTWARE: FastSEQ Version 1.5
- (vi) ATTORNEY/AGENT INFORMATION:
- 35 (A) NAME: Fisher, Carlos A
(B) REGISTRATION NUMBER: 36,510
(C) REFERENCE/DOCKET NUMBER: GP94013
- (vii) TELECOMMUNICATION INFORMATION:
- 40 (A) TELEPHONE: 619-535-2807
(B) TELEFAX: 619-546-7929

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCTCGTTGCG GGACTTAACC CAACAT

26

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGTTGGGTT AAGTCCCGCA ACGAGC

26

WHAT IS CLAIMED IS:

1. A method of increasing the hybridization rate between first and second nucleic acids in a diagnostic hybridization assay for use in detecting the presence or amount of at least one nucleic acid analyte, wherein a first nucleotide base sequence region of said first nucleic acid is able to stably hybridize to a first nucleotide base sequence region of said second nucleic acid under selective hybridization conditions, which comprises the steps of:
- 5 a) synthesizing at least one of said nucleotide regions to include one or more modified nucleotides, so that
- 10 i) the hybridization binding affinity between said first and second nucleic acids is greater than the hybridization binding affinity between unmodified forms of said first and second nucleic acids, under said conditions, and
- ii) the hybridization rate between said first and second nucleic acids is greater than the hybridization rate between unmodified forms of said first and second nucleic acids, under said conditions; and
- 15 b) contacting said first and second nucleic acids of step a) under said conditions, such that said nucleotide regions are able to stably hybridize.
2. The method of claim 1, wherein said first and second nucleic acids are
- 20 contained on the same nucleic acid strand.
3. The method of claim 1, wherein at least one of said nucleotide regions includes one or more clusters of at least about 4 modified nucleotides.
- 25 4. The method of claim 1, wherein at least one of said nucleotide regions includes one or more clusters of at least about 6 modified nucleotides.
5. The method of claim 1, wherein at least one of said nucleotide regions

includes one or more clusters of at least about 8 modified nucleotides.

6. The method of claim 1, wherein substantially all of the nucleotides contained in at least one of said nucleotide regions are modified.

7. The method of claim 3, wherein at least one of said modified nucleotides includes a modification selected from the group consisting of:

- a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;
- c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage; and
- e) a modification to the internucleotide linkage.

8. The method of claim 3, wherein at least one of said modified nucleotides includes two modifications selected from the group consisting of:

- a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;
- c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage;
- e) a modification to the internucleotide linkage.

9. The method of claim 3, wherein at least one of said modified nucleotides includes a 2'-modification to the ribofuranosyl moiety selected from the group consisting of:

- a) an alkyl substitution;
- b) an alkoxy substitution; and
- c) a halide substitution.

10. The method of claim 3, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

5 11. The method of claim 3, wherein at least one of said modified nucleotides includes a propyne substitution to the nitrogenous base.

12. The method of claim 11, wherein said propyne substitution is to a cytidine analog.

10 13. The method of claim 11, wherein said propyne substitution is to a thymidine analog.

14. The method of claim 3, wherein each said modified nucleotide of one or more of said clusters of at least one of said nucleotide regions contains the same
15 modification.

15. The method of claim 14, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

20 16. The method of claim 1, wherein at least one of said nucleic acids includes one or more conjugate molecules.

25 17. The method of claim 3, wherein at least one of said nucleic acids includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said nucleic acids at a site located within one or more of said clusters contained in at least one of said nucleotide regions.

18. The method of claim 3, wherein said first nucleotide region of said first

nucleic acid includes one or more clusters of at least about 4 modified nucleotides.

19. The method of claim 18, wherein said first nucleic acid comprises an oligonucleotide probe.

20. The method of claim 19, wherein said probe consists of from about 10 to about 100 nucleotide bases.

21. The method of claim 19, wherein said probe consists of from about 10 to about 15 nucleotide bases.

22. The method of claim 19, wherein said probe consists of from about 12 to about 15 nucleotide bases.

23. The method of claim 19, wherein said probe further includes a label.

24. The method of claim 23, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b) an enzyme,
- c) an enzyme cofactor,
- d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- h) a fluorescent molecule,
- i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule.

k) a chromophore, and
l) a nucleotide base sequence region that is unable to stably hybridize to said second nucleic acid under said conditions.

5 25. The method of claim 24, wherein said label is a chemiluminescent molecule.

 26. The method of claim 25, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.
10

 27. The method of claim 25, wherein said chemiluminescent molecule is an acridinium ester derivative.

15 28. The method of claim 23, wherein said label is joined to said probe at a site located within one of said clusters contained in said first nucleotide region of said probe.

 29. The method of claim 19, wherein said second nucleic acid comprises said analyte.
20

 30. The method of claim 29, wherein said analyte consists of RNA.

 31. The method of claim 30, wherein said RNA consists of rRNA or tRNA.

25 32. The method of claim 29, wherein said analyte consists of DNA.

 33. The method of claim 3, wherein each said nucleotide region includes one or more clusters of at least about 4 modified nucleotides.

34. The method of claim 33, wherein each said modified nucleotide of one or more of said clusters of at least one of said nucleotide regions contains the same modification.

5 35. The method of claim 34, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

36. A method for ~~detecting the presence~~ or amount of an analyte comprising a first nucleic acid in a ~~sample suspected of containing~~ said analyte, which
10 comprises the steps of:

a) contacting said sample with a probe comprising a second nucleic acid, wherein said probe contains a first nucleotide base sequence region that is able to stably hybridize to a first nucleotide base sequence region of said analyte under selective hybridization conditions, and wherein said first nucleotide region of said probe includes one
15 or more modified nucleotides;

b) subjecting the components of step a) to said conditions, so that
i) the hybridization binding affinity between said analyte and said probe is greater than the hybridization binding affinity between said analyte and an unmodified form of said probe, under said conditions, and

20 ii) the hybridization rate between said analyte and said probe is greater than the hybridization rate between said analyte and an unmodified form of said probe, under said conditions; and

c) detecting said probe hybridized to said analyte as an indication of the presence or amount of said analyte in said sample.

25 37. The method of claim 36, wherein said first nucleotide region of said probe includes one or more clusters of at least about 4 modified nucleotides.

38. The method of claim 36, wherein said first nucleotide region of said probe includes one or more clusters of at least about 6 modified nucleotides.

39. The method of claim 36, wherein said first nucleotide region of said probe includes one or more clusters of at least about 8 modified nucleotides.

40. The method of claim 36, wherein substantially all of the nucleotides contained in said first nucleotide region of said probe are modified.

41. The method of claim 37, wherein at least one of said modified nucleotides includes a modification selected from the group consisting of:

- a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;
- c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage; and
- e) a modification to the internucleotide linkage.

42. The method of claim 37, wherein at least one of said modified nucleotides includes two modifications selected from the group consisting of:

- a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;
- c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage; and
- e) a modification to the internucleotide linkage.

43. The method of claim 37, wherein at least one of said modified nucleotides includes a 2'-modification to the ribofuranosyl moiety selected from the group consisting of:

- a) an alkyl substitution;
- b) an alkoxy substitution; and
- c) a halide substitution.

5 44. The method of claim 37, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

 45. The method of claim 37, wherein at least one of said modified nucleotides includes a propyne substitution to the nitrogenous base.

10 46. The method of claim 45, wherein said propyne substitution is to a cytidine analog.

 47. The method of claim 45, wherein said propyne substitution is to a
15 thymidine analog.

 48. The method of claim 37, wherein each said modified nucleotide of one or more of said clusters contains the same modification.

20 49. The method of claim 48, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

 50. The method of claim 36, wherein said probe includes one or more conjugate molecules.

25 51. The method of claim 37, wherein said probe includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to said probe at a site located within one or more of said clusters contained in said first nucleotide

region of said probe.

52. The method of claim 37, wherein said probe is an oligonucleotide consisting of from about 10 to about 100 nucleotide bases.

5

53. The method of claim 37, wherein said probe is an oligonucleotide consisting of from about 10 to about 15 nucleotide bases.

54. The method of claim 37, wherein said probe is an oligonucleotide consisting of from about 12 to about 15 nucleotide bases.

10

55. The method of claim 37, wherein said probe further includes a label.

56. The method of claim 55, wherein said label is selected from the group consisting of:

15

- a) a radioisotope,
- b) an enzyme,
- c) an enzyme cofactor,
- d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- h) a fluorescent molecule,
- i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- l) a nucleotide base sequence region that is unable to stably

20

25

hybridize to said analyte under said conditions.

57. The method of claim 56, wherein said label is a chemiluminescent molecule.

58. The method of claim 57, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

59. The method of claim 57, wherein said chemiluminescent molecule is an acridinium ester derivative.

60. The method of claim 55, wherein said label is joined to said probe at a site located within one of said clusters contained in said first nucleotide region of said probe.

61. The method of claim 37, wherein said analyte consists of RNA.

62. The method of claim 61, wherein said RNA is rRNA or tRNA.

63. The method of claim 61, wherein said sample further contains DNA.

64. The method of claim 37, wherein said analyte consists of DNA.

65. The method of either claim 37 or 55, wherein said probe or said analyte is directly or indirectly immobilized by a solid support.

66. The method of claim 36, wherein said contacting step further includes contacting said probe with a third nucleic acid, wherein said third nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said probe under selective hybridization conditions.

wherein said analyte is unable to stably hybridize to either said third nucleic acid or said second nucleotide region of said probe under said conditions, and

wherein said third nucleic acid is unable to stably hybridize to said first nucleotide region of said probe under said conditions.

5

67. The method of claim 66, wherein at least one of said nucleotide regions of said probe and said third nucleic acid includes one or more clusters of at least about 4 modified nucleotides.

10

68. The method of claim 66, wherein substantially all of the nucleotides contained in at least one of said nucleotide regions of said probe and said third nucleic acid are modified.

15

69. The method of claim 67, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

20

70. The method of claim 67, wherein each said modified nucleotide of one or more of said clusters of at least one of said nucleotide regions of said probe and said third nucleic acid contains the same modification.

25

71. The method of claim 70, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

72. The method of claim 66, wherein at least one of said probe and said third nucleic acid includes one or more conjugate molecules.

73. The method of claim 67, wherein at least one of said probe and said third nucleic acid includes one or more conjugate molecules, and wherein at least one of said

conjugate molecules is joined to at least one of said probe and said third nucleic acid at a site located within one or more of said clusters contained in at least one of said nucleotide regions of said probe and said third nucleic acid.

5 74. The method of claim 67, wherein said probe further includes a label.

75. The method of claim 74, wherein said label is selected from the group consisting of:

- 10 a) a radioisotope,
 b) an enzyme,
 c) an enzyme cofactor,
 d) an enzyme substrate,
 e) a dye,
 f) a hapten,
15 g) a chemiluminescent molecule,
 h) a fluorescent molecule,
 i) a phosphorescent molecule,
 j) an electrochemiluminescent molecule,
 k) a chromophore, and
20 l) a nucleotide base sequence region that is unable to stably
hybridize to either said analyte or said third nucleic acid under said conditions.

76. The method of claim 75, wherein said label is a chemiluminescent molecule.

25 77. The method of claim 76, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

78. The method of claim 76, wherein said label is an acridinium ester derivative.

79. The method of claim 67, wherein said label is joined to said probe at a site located within one of said clusters contained in said first nucleotide region of said probe.

80. The method of claim 67, wherein said analyte consists of RNA.

81. The method of either claim 67 or 74, wherein one of said analyte and said third nucleic acid is directly or indirectly immobilized by a solid support.

82. The method of claim 36, wherein said contacting step further includes contacting said analyte with a third nucleic acid, wherein said third nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under selective hybridization conditions,

wherein said probe is unable to stably hybridize to either said third nucleic acid or said second nucleotide region of said analyte under said conditions, and

wherein said third nucleic acid is unable to stably hybridize to said first nucleotide region of said analyte under said conditions.

83. The method of claim 82, wherein at least one of said nucleotide regions of said probe and said third nucleic acid includes one or more clusters of at least about 4 modified nucleotides.

84. The method of claim 82, wherein substantially all of the nucleotides contained in at least one of said nucleotide regions of said probe and said third nucleic acid are modified.

85. The method of claim 83, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

86. The method of claim 83, wherein each said modified nucleotide of one or more of said clusters of at least one of said nucleotide regions of said probe and said third nucleic acid contains the same modification.

87. The method of claim 86, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

88. The method of claim 82, wherein at least one of said probe and said third nucleic acid includes one or more conjugate molecules.

89. The method of claim 83, wherein at least one of said probe and said third nucleic acid includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said probe and said third nucleic acid at a site located within one or more of said clusters contained in at least one of said nucleotide regions of said probe and said third nucleic acid.

90. The method of claim 83, wherein said third nucleic acid is a helper probe.

91. The method of claim 83, wherein said probe further includes a label.

92. The method of claim 91, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b) an enzyme,

- 5
- c) an enzyme cofactor,
 - d) an enzyme substrate,
 - e) a dye,
 - f) a hapten,
 - g) a chemiluminescent molecule,
 - h) a fluorescent molecule,
 - i) a phosphorescent molecule,
 - j) an electrochemiluminescent molecule,
 - k) a chromophore, and
 - 10 l) a nucleotide base sequence region that is unable to stably hybridize to either said analyte or said third nucleic acid under said conditions.

15 93. The method of claim 92, wherein said label is a chemiluminescent molecule.

94. The method of claim 93, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

20 95. The method of claim 93, wherein said label is an acridinium ester derivative.

96. The method of claim 91, wherein said label is joined to said probe at a site located within one of said clusters contained in said first nucleotide region of said probe.

25 97. The method of claim 83, wherein said analyte consists of RNA.

98. The method of either claim 83 or 91, wherein one of said probe and

said third nucleic acid is directly or indirectly immobilized by a solid support.

5 99. The method of claim 36, wherein said contacting step further includes contacting a third nucleic acid with said analyte and said probe, wherein said third nucleic acid contains:

a) a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under selective hybridization conditions; and

10 b) a second nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said probe under selective hybridization conditions,

wherein said analyte is not able to stably hybridize to either said second nucleotide region of said probe or said second nucleotide region of said third nucleic acid under said conditions,

15 wherein said probe is unable to stably hybridize to either said second nucleotide region of said analyte or said first nucleotide region of said third nucleic acid under said conditions, and

wherein said third nucleic acid is unable to stably hybridize to either said first nucleotide region of said analyte or said first nucleotide region of said probe.

20 100. The method of claim 99, wherein at least one of said nucleotide regions of said probe and said third nucleic acid includes one or more clusters of at least about 4 modified nucleotides.

25 101. The method of claim 99, wherein substantially all of the nucleotides contained in at least one of said nucleotide regions of said probe and said third nucleic acid are modified.

102. The method of claim 100, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

5 103. The method of claim 100, wherein each said modified nucleotide of one or more of said clusters of at least one of said nucleotide regions of said probe and said third nucleic acid contains the same modification.

10 104. The method of claim 103, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

105. The method of claim 99, wherein at least one of said probe and said third nucleic acid includes one or more conjugate molecules.

15 106. The method of claim 100, wherein at least one of said probe and said third nucleic acid includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said probe and said third nucleic acid at a site located within one or more of said clusters contained in at least one of said nucleotide regions of said probe and said third nucleic acid.

20 107. The method of claim 100, wherein said probe further includes a label.

108. The method of claim 107, wherein said label is selected from the group consisting of:

- 25
- a) a radioisotope,
 - b) an enzyme,
 - c) an enzyme cofactor,
 - d) an enzyme substrate,
 - e) a dye.

- f) a hapten,
- g) a chemiluminescent molecule,
- h) a fluorescent molecule,
- i) a phosphorescent molecule,
- 5 j) an electrochemiluminescent molecule,
- k) a chromophore, and
- l) a nucleotide base sequence region that is unable to stably

hybridize to either said analyte or said third nucleic acid under said conditions.

10 109. The method of claim 108, wherein said label is a chemiluminescent molecule.

110. The method of claim 109, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.
15

111. The method of claim 109, wherein said label is an acridinium ester derivative.

20 112. The method of claim 100, wherein said analyte consists of RNA.

113. The method of either claim 100 or 107, wherein one of said probe and said third nucleic acid is directly or indirectly immobilized by a solid support.

25 114. The method of claim 36, wherein said contacting step further includes contacting the following components:

- a) said probe with a third nucleic acid, wherein said third nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second

nucleotide base sequence region of said probe under selective hybridization conditions; and

b) said third nucleic acid with a fourth nucleic acid, wherein said fourth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said third nucleic acid under selective hybridization conditions.

wherein said analyte is unable to stably hybridize to any of said second nucleotide region of said probe and said third and fourth nucleic acids under said conditions,

wherein said probe is unable to stably hybridize to either of said second nucleotide region of said third nucleic acid and said fourth nucleic acid under said conditions,

wherein said third nucleic acid is unable to stably hybridize to said first nucleotide region of said probe under said conditions, and

wherein said fourth nucleic acid is unable to stably hybridize to said first nucleotide region of said third nucleic acid under said conditions.

115. The method of claim 114, wherein at least one of said nucleotide regions of said probe and said third and fourth nucleic acids includes one or more clusters of at least about 4 modified nucleotides.

116. The method of claim 114, wherein substantially all of the nucleotides contained in at least one of said nucleotide regions of said probe and said third and fourth nucleic acids are modified.

117. The method of claim 115, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

118. The method of claim 115, wherein each said modified nucleotide of one or more of said clusters of at least one of said probe and said third and fourth nucleic acids contains the same modification.

119. The method of claim 118, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

120. The method of claim 114, wherein at least one of said probe and said third and fourth nucleic acids includes one or more conjugate molecules.

121. The method of claim 115, wherein at least one of said probe and said third and fourth nucleic acids includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said probe and said third and fourth nucleic acids at a site located within one or more of said clusters contained in at least one of said nucleotide regions of said probe and said third and fourth nucleic acids.

122. The method of claim 115, wherein said probe further includes a label.

123. The method of claim 122, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b) an enzyme,
- c) an enzyme cofactor,
- d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- h) a fluorescent molecule,
- i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- l) a nucleotide base sequence region that is unable to stably

hybridize to any of said analyte and said third and fourth nucleic acids under said conditions.

124. The method of claim 123, wherein said label is a chemiluminescent molecule.

125. The method of claim 124, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

126. The method of claim 124, wherein said label is an acridinium ester derivative.

127. The method of claim 122, wherein said label is joined to said probe at a site located within one of said clusters of said first nucleotide region of said probe.

128. The method of claim 115, wherein said analyte consists of RNA.

129. The method of either claim 115 or 122, wherein said fourth nucleic acid is immobilized by a solid support.

130. The method of claim 36, wherein said contacting step further includes contacting the following components:

a) said analyte with a third nucleic acid, wherein said third nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under selective hybridization conditions; and

b) said third nucleic acid with a fourth nucleic acid, wherein said fourth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said third nucleic acid under selective

hybridization conditions,

wherein said analyte is unable to stably hybridize to either said second nucleotide region of said third nucleic acid or said fourth nucleic acid under said conditions,

5 wherein said probe is unable to stably hybridize to any of said second nucleotide region of said analyte and said third and fourth nucleic acids under said conditions,

wherein said third nucleic acid is unable to stably hybridize to either said first nucleotide region of said analyte or said probe under said conditions, and

wherein said fourth nucleic acid is unable to stably hybridize to said first nucleotide region of said third nucleic acid under said conditions.

10

131. The method of claim 130, wherein at least one of said nucleotide regions of said probe and said third and fourth nucleic acids includes one or more clusters of at least about 4 modified nucleotides.

15

132. The method of claim 130, wherein substantially all of the nucleotides contained in at least one of said nucleotide regions of said probe and said third and fourth nucleic acids are modified.

20

133. The method of claim 131, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

25

134. The method of claim 131, wherein each said modified nucleotide of one or more of said clusters of at least one of said nucleotide regions of said probe and said third and fourth nucleic acids contains the same modification.

135. The method of claim 134, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

136. The method of claim 130, wherein at least one of said probe and said third and fourth nucleic acids includes one or more conjugate molecules.

137. The method of claim 131, wherein at least one of said probe and said third and fourth nucleic acids includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said probe and said third and fourth nucleic acids at a site located within one or more of said clusters contained in at least one of said nucleotide regions of said probe and said third and fourth nucleic acids.

138. The method of claim 131, wherein said probe further includes a label.

139. The method of claim 138, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b) an enzyme,
- c) an enzyme cofactor,
- d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- h) a fluorescent molecule,
- i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- l) a nucleotide base sequence region that is unable to stably

hybridize to any of said analyte and said third and fourth nucleic acids under said conditions.

140. The method of claim 139, wherein said label is a chemiluminescent

molecule.

141. The method of claim 140, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

142. The method of claim 140, wherein said label is an acridinium ester derivative.

143. The method of claim 138, wherein said label is joined to said probe at a site located within one of said clusters of said first nucleotide region of said probe.

144. The method of claim 131, wherein said analyte consists of RNA.

145. The method of either claim 131 or 138, wherein said fourth nucleic acid is immobilized by a solid support.

146. The method of claim 36, wherein said contacting step further includes contacting the following components:

a) said analyte with a third nucleic acid, wherein said third nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under selective hybridization conditions;

b) said probe with said third nucleic acid, wherein said third nucleic acid contains a second nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said probe under selective hybridization conditions; and

c) said third nucleic acid with a fourth nucleic acid, wherein said fourth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a third nucleotide base sequence region of said third nucleic acid under selective hybridization

conditions.

wherein said analyte is unable to stably hybridize to any of said second nucleotide region of said probe, said second and third nucleotide regions of said third nucleic acid, and said fourth nucleic acid *under* said conditions,

5 wherein said probe is unable to stably hybridize to any of said second nucleotide region of said analyte, said first and third nucleotide regions of said third nucleic acid, and said fourth nucleic acid under said conditions,

 wherein said third nucleic acid is unable to stably hybridize to either said first nucleotide region of said analyte or said first nucleotide region of said probe under said
10 conditions, and

 wherein said fourth nucleic acid is *unable* to stably hybridize to either said first or second nucleotide region of said third nucleic acid under said conditions.

147. The method of claim 146, wherein at least one of said nucleotide
15 regions of said probe and said third and fourth nucleic acids includes one or more clusters of at least about 4 modified nucleotides.

148. The method of claim 146, wherein substantially all of the nucleotides
20 contained in at least one of said nucleotide regions of said probe and said third and fourth nucleic acids are modified.

149. The method of claim 147, wherein at least one of said modified
nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

25 150. The method of claim 147, wherein each said modified nucleotide of one or more of said clusters of at least one of said nucleotide regions of said probe and said third and fourth nucleic acids contains the same modification.

151. The method of claim 150, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

5 152. The method of claim 146, wherein at least one of said probe and said third and fourth nucleic acids includes one or more conjugate molecules.

10 153. The method of claim 147, wherein at least one of said probe and said third and fourth nucleic acids includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said probe and said third and fourth nucleic acids at a site located within one or more of said clusters contained in at least one of said nucleotide regions of said probe and said third and fourth nucleic acids.

154. The method of claim 147, wherein said probe further includes a label.

15 155. The method of claim 154, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b) an enzyme,
- c) an enzyme cofactor,
- 20 d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- h) a fluorescent molecule,
- 25 i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- l) a nucleotide base sequence region that is unable to stably

hybridize to any of said analyte and said third and fourth nucleic acids under said conditions.

156. The method of claim 155, wherein said label is a chemiluminescent molecule.

5

157. The method of claim 156, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

10

158. The method of claim 156, wherein said label is an acridinium ester derivative.

15

159. The method of claim 154, wherein said label is joined to said probe at a site located within one of said clusters of said first nucleotide region of said probe.

160. The method of claim 147, wherein said analyte consists of RNA.

20

161. The method of either claim 147 or 154, wherein said fourth nucleic acid is immobilized by a solid support.

162. The method of claim 36, wherein said contacting step further includes contacting the following components:

25

a) said probe with a first coupling nucleic acid comprising a third nucleic acid, wherein said first coupling nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said probe under selective hybridization conditions;

b) a second coupling nucleic acid comprising a fourth nucleic acid with a fifth nucleic acid, wherein said fifth nucleic acid contains a first nucleotide base

sequence region able to stably hybridize to a first nucleotide base sequence region of said second coupling nucleic acid under selective hybridization conditions; and, alternatively,

5 i) said first coupling nucleic acid with said second coupling nucleic acid, wherein said second coupling nucleic acid contains a second nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said first coupling nucleic acid under selective hybridization conditions; or

10 ii) one or more optional coupling nucleic acids, other than said first and second coupling nucleic acids, wherein each of said optional coupling nucleic acids can stably hybridize to at least two other coupling nucleic acids under selective hybridization conditions, wherein at least one of said other coupling nucleic acids may be at least one of said first and second coupling nucleic acids, and wherein each of said other coupling nucleic acids is directly or indirectly joined to said fifth nucleic acid and said analyte,

15 wherein said analyte is unable to stably hybridize to any of said second nucleotide region of said probe, said first and second coupling nucleic acids, said fifth nucleic acid, and said optional coupling nucleic acids under said conditions,

wherein said probe is unable to stably hybridize to any of said second nucleotide region of said first coupling nucleic acid, said second coupling nucleic acid, said fifth nucleic acid, and said optional coupling nucleic acids under said conditions,

20 wherein said first coupling nucleic acid is unable to stably hybridize to any of said first nucleotide region of said probe, said first nucleotide region of said second coupling nucleic acid, and said fifth nucleic acid under said conditions,

wherein said second coupling nucleic acid is unable to stably hybridize to said first nucleotide region of said first coupling nucleic acid under said conditions,

25 wherein said fifth nucleic acid is unable to stably hybridize to either said second nucleotide region of said second coupling nucleic acid or said optional coupling nucleic acids under said conditions, and

wherein said optional coupling nucleic acids do not stably hybridize to said

first nucleotide region of either said first or second coupling nucleic acid under said conditions.

5 163. The method of claim 162, wherein at least one of said nucleotide regions of said probe, said first and second coupling nucleic acids, said fifth nucleic acid, and a nucleotide base sequence region of any one of said optional coupling nucleic acids includes one or more clusters of at least about 4 modified nucleotides.

10 164. The method of claim 162, wherein substantially all of the nucleotides contained in at least one of said nucleotide regions of said probe, said first and second coupling nucleic acids, said fifth nucleic acid, and a nucleotide base sequence region of any one of said optional coupling nucleic acids are modified.

15 165. The method of claim 163, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

20 166. The method of claim 163, wherein each said modified nucleotide of one or more of said clusters of at least one of said nucleotide regions of said probe, said fifth nucleic acid, and any of said coupling nucleic acids contains the same modification.

167. The method of claim 166, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

25 168. The method of claim 162, wherein at least one of said probe, said fifth nucleic acid, and any of said coupling nucleic acids includes one or more conjugate molecules.

169. The method of claim 163, wherein at least one of said probe, said fifth

nucleic acid, and any of said coupling nucleic acids includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said probe, said fifth nucleic acid, and any of said coupling nucleic acids at a site located within one or more of said clusters contained in at least one of said nucleotide regions of said probe, said fifth nucleic acid, and any of said coupling nucleic acids.

170. The method of claim 163, wherein said probe further includes a label.

171. The method of claim 170, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b) an enzyme,
- c) an enzyme cofactor,
- d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- h) a fluorescent molecule,
- i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- l) a nucleotide base sequence region that is unable to stably

hybridize to any of said analyte, said fifth nucleic acid, and said coupling nucleic acids under said conditions.

172. The method of claim 171, wherein said label is a chemiluminescent molecule.

173. The method of claim 172, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

5 174. The method of claim 172, wherein said label is an acridinium ester derivative.

175. The method of claim 170, wherein said label is joined to said probe at a site located within one of said clusters contained in said first nucleotide region of said probe.

10 176. The method of claim 163, wherein said analyte consists of RNA.

177. The method of either claim 163 or 170, wherein said fifth nucleic acid is immobilized by a solid support.

15 178. The method of claim 36, wherein said contacting step further includes contacting the following components:

a) said analyte with a first coupling nucleic acid comprising a third nucleic acid, wherein said first coupling nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under selective hybridization conditions;

20 b) a second coupling nucleic acid comprising a fourth nucleic acid with a fifth nucleic acid, wherein said fifth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said second coupling nucleic acid under selective hybridization conditions; and, alternatively,

25 i) said first coupling nucleic acid with said second coupling nucleic acid, wherein said second coupling nucleic acid contains a second nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said

first coupling nucleic acid under selective hybridization conditions; or

ii) one or more optional coupling nucleic acids, other than said first and second coupling nucleic acids, wherein each of said optional coupling nucleic acids is able to stably hybridize to two other coupling nucleic acids under selective hybridization conditions, wherein at least one of said other coupling nucleic acids may be at least one of said first and second coupling nucleic acids, and wherein each of said other coupling nucleic acids is directly or indirectly joined to said fifth nucleic acid and said analyte,

wherein said analyte is unable to stably hybridize to any of said second nucleotide region of said first coupling nucleic acid, said second coupling nucleic acid, said fifth nucleic acid, and said optional coupling nucleic acids under said conditions,

wherein said probe is unable to stably hybridize to any of said second nucleotide region of said analyte, said first and second coupling nucleic acids, said fifth nucleic acid, and said optional coupling nucleic acids under said conditions,

wherein said first coupling nucleic acid is unable to stably hybridize to any of said first nucleotide region of said analyte, said first nucleotide region of said second coupling nucleic acid, and said fifth nucleic acid under said conditions,

wherein said second coupling nucleic acid is unable to stably hybridize to said first nucleotide region of said first coupling nucleic acid under said conditions,

wherein said fifth nucleic acid is unable to stably hybridize to either said second nucleotide region of said second coupling nucleic acid or said optional coupling nucleic acids under said conditions, and

wherein said optional coupling nucleic acids do not stably hybridize to said first nucleotide region of either said first or second coupling nucleic acid under said conditions.

179. The method of claim 178, wherein at least one of said nucleotide regions of said probe, said first and second coupling nucleic acids, said fifth nucleic acid, and

a nucleotide base sequence region of any one of said optional coupling nucleic acids includes one or more clusters of at least about 4 modified nucleotides.

5 180. The method of claim 178, wherein substantially all of the nucleotides contained in at least one of said nucleotide regions of said probe, said first and second coupling nucleic acids, said fifth nucleic acid, and a nucleotide base sequence region of any one of said optional coupling nucleic acids are modified.

10 181. The method of claim 179, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

15 182. The method of claim 179, wherein each said modified nucleotide of one or more of said clusters of at least one of said nucleotide regions of said probe, said fifth nucleic acid, and any of said coupling nucleic acids contains the same modification.

183. The method of claim 182, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

20 184. The method of claim 178, wherein at least one of said probe, said fifth nucleic acid, and any of said coupling nucleic acids includes one or more conjugate molecules.

25 185. The method of claim 179, wherein at least one of said probe, said fifth nucleic acid, and any of said coupling nucleic acids includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said probe, said fifth nucleic acid, and any of said coupling nucleic acids at a site located within one or more of said clusters contained in at least one of said nucleotide regions of said probe, said fifth nucleic acid, and any of said coupling nucleic acids.

186. The method of claim 179, wherein said probe further includes a label.

187. The method of claim 186, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b) an enzyme,
- c) an enzyme cofactor,
- d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- h) a fluorescent molecule,
- i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- l) a nucleotide base sequence region that is unable to stably

hybridize to any of said analyte, said fifth nucleic acid, and any of said coupling nucleic acids under said conditions.

188. The method of claim 187, wherein said label is a chemiluminescent molecule.

189. The method of claim 188, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

190. The method of claim 188, wherein said label is an acridinium ester derivative.

191. The method of claim 186, wherein said label is joined to said probe at a site located within said first nucleotide region of said probe.

192. The method of claim 179, wherein said analyte consists of RNA.

193. The method of either claim 179 or 186, wherein said fifth nucleic acid is immobilized by a solid support.

194. The method of claim 36, wherein said contacting step further includes contacting the following components:

a) said probe with a first coupling nucleic acid comprising a third nucleic acid, wherein said first coupling nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said probe under selective hybridization conditions;

b) said analyte with said first coupling nucleic acid, wherein said first coupling nucleic acid contains a second nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under selective hybridization conditions;

c) a second coupling nucleic acid comprising a fourth nucleic acid with a fifth nucleic acid, wherein said fifth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said second coupling nucleic acid under selective hybridization conditions; and, alternatively,

i) said first coupling nucleic acid with said second coupling nucleic acid, wherein said second coupling nucleic acid contains a second nucleotide base sequence region able to stably hybridize to a third nucleotide base sequence region of said first coupling nucleic acid under selective hybridization conditions; or

ii) one or more optional coupling nucleic acids, other than said first and second coupling nucleic acids, wherein each of said optional coupling nucleic

acids is able to stably hybridize to two other coupling nucleic acids under selective hybridization conditions, wherein at least one of said other coupling nucleic acids may be at least one of said first and second coupling nucleic acids, and wherein each of said other coupling nucleic acids is directly or indirectly joined to said analyte, said probe and said fifth nucleic acid,

wherein said analyte is unable to stably hybridize to any of said second nucleotide region of said probe, said first and third nucleotide regions of said first coupling nucleic acid, said second coupling nucleic acid, said fifth nucleic acid, and said optional coupling nucleic acids under said conditions,

wherein said probe is unable to stably hybridize to any of said second nucleotide region of said analyte, said second and third nucleotide regions of said first coupling nucleic acid, said second coupling nucleic acid, said fifth nucleic acid, and said optional coupling nucleic acids under said conditions,

wherein said first coupling nucleic acid is unable to stably hybridize to any of said first nucleotide region of said analyte, said first nucleotide region of said probe, said first nucleotide region of said second coupling nucleic acid, and said fifth nucleic acid under said conditions,

wherein said second coupling nucleic acid is unable to stably hybridize to either said first or second nucleotide region of said first coupling nucleic acid under said conditions,

wherein said fifth nucleic acid is unable to stably hybridize to either said second nucleotide region of said second coupling nucleic acid or said optional coupling nucleic acids under said conditions, and

wherein said optional coupling nucleic acids do not stably hybridize to any of said first and second nucleotide regions of said first coupling nucleic acid and said first nucleotide region of said second coupling nucleic acid under said conditions.

195. The method of claim 194, wherein at least one of said nucleotide

regions of said probe, said first and second coupling nucleic acids, said fifth nucleic acid, and a nucleotide base sequence region of any of said optional coupling nucleic acids includes one or more clusters of at least about 4 modified nucleotides.

5 196. The method of claim 194, wherein substantially all of the nucleotides contained in at least one of said nucleotide regions of said probe, said first and second coupling nucleic acids, said fifth nucleic acid, and a nucleotide base sequence region of any of said optional coupling nucleic acids are modified.

10 197. The method of claim 195, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

 198. The method of claim 195, wherein each said modified nucleotide of one or more of said clusters of at least one of said nucleotide regions of said probe, said fifth
15 nucleic acid, and any of said coupling nucleic acids contains the same modification.

 199. The method of claim 198, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

20 200. The method of claim 194, wherein at least one of said probe, said fifth nucleic acid, and any of said coupling nucleic acids includes one or more conjugate molecules.

 201. The method of claim 195, wherein at least one of said probe, said fifth
25 nucleic acid, and any of said coupling nucleic acids includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said probe, said fifth nucleic acid, and any of said coupling nucleic acids at a site located within one or more of said clusters contained in at least one of said nucleotide regions of said

probe, said fifth nucleic acid, and any of said coupling nucleic acids.

202. The method of claim 195, wherein said probe further includes a label.

5 203. The method of claim 202, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b) an enzyme,
- c) an enzyme cofactor,
- 10 d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- h) a fluorescent molecule,
- 15 i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- l) a nucleotide base sequence region that is unable to stably

20 hybridize to any of said analyte, said fifth nucleic acid, and said coupling nucleic acids under said conditions.

204. The method of claim 203, wherein said label is a chemiluminescent molecule.

25 205. The method of claim 204, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

206. The method of claim 204, wherein said label is an acridinium ester derivative.

207. The method of claim 202, wherein said label is joined to said probe at a
5 site located within said first nucleotide region of said probe.

208. The method of claim 195, wherein said analyte consists of RNA.

209. The method of either claim 195 or 202, wherein said fifth nucleic acid
10 is immobilized by a solid support.

210. A method for detecting the presence or amount of an analyte comprising a first nucleic acid in a sample suspected of containing said analyte, which comprises the steps of:

15 a) contacting the following components:

i) said sample with a second nucleic acid, wherein said second nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said analyte under selective hybridization conditions; and

20 ii) a probe comprising a third nucleic acid with said second nucleic acid, wherein said second nucleic acid contains a second nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said probe under selective hybridization conditions,

25 wherein said second nucleic acid cannot stably hybridize to said probe unless said second nucleic acid is stably hybridized with said analyte,

wherein said analyte is unable to stably hybridize to either said probe or said second nucleotide region of said second nucleic acid under said conditions,

wherein said probe is unable to stably hybridize to said first nucleotide region

of said second nucleic acid under said conditions.

wherein said second nucleic acid is unable to stably hybridize to said first nucleotide region of said analyte under said conditions, and

wherein at least one of said nucleotide regions of said probe and said second nucleic acid includes one or more modified nucleotides;

b) subjecting the components of step a) to selective hybridization conditions, so that

if at least one of said first nucleotide region of said probe and said second nucleotide region of said second nucleic acid includes one or more of said modified nucleotides, then

i) the hybridization binding affinity between said probe and said second nucleic acid is greater than the hybridization binding affinity between unmodified forms of said probe and said second nucleic acid, under identical hybridization conditions, and

ii) the hybridization rate between said probe and said second nucleic acid is greater than the hybridization rate between unmodified forms of said probe and said second nucleic acid, under identical hybridization conditions, and

if said first nucleotide region of said second nucleic acid includes one or more of said modified nucleotides, then

i) the hybridization binding affinity between said analyte and said second nucleic acid is greater than the hybridization binding affinity between said analyte and an unmodified form of said second nucleic acid, under identical hybridization conditions, and

ii) the hybridization rate between said analyte and said second nucleic acid is greater than the hybridization rate between said analyte and an unmodified form of said second nucleic acid, under identical hybridization conditions; and

c) detecting said labeled probe hybridized with said second nucleic acid as an indication of the presence or amount of said analyte in said sample.

PCT/US 97/12347

211. The method of claim 210, wherein at least one of said nucleotide regions of said probe and said second nucleic acid includes one or more clusters of at least about 4 modified nucleotides.

5 212. The method of claim 210, wherein at least one of said nucleotide regions of said probe and said second nucleic acid includes one or more clusters of at least about 6 modified nucleotides.

10 213. The method of claim 210, wherein at least one of said nucleotide regions of said probe and said second nucleic acid includes one or more clusters of at least about 8 modified nucleotides.

15 214. The method of claim 210, wherein substantially all of the nucleotides contained in at least one of said nucleotide regions of said probe and said second nucleic acid are modified.

20 215. The method of claim 211, wherein at least one of said modified nucleotides includes a modification selected from the group consisting of:

- a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;
- c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage; and
- e) a modification to the internucleotide linkage.

25 216. The method of claim 211, wherein at least one of said modified nucleotides includes two modifications selected from the group consisting of:

- a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;

- c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage; and
- e) a modification to the internucleotide linkage.

5 217. The method of claim 211, wherein at least one of said modified nucleotides includes a 2'-modification to the ribofuranosyl moiety selected from the group consisting of:

- a) an alkyl substitution;
- b) an alkoxy substitution; and
- 10 c) a halide substitution.

 218. The method of claim 211, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

15 219. The method of claim 211, wherein at least one of said modified nucleotides includes a propyne substitution to the nitrogenous base.

 220. The method of claim 219, wherein said propyne substitution is to a cytidine analog.

20 221. The method of claim 219, wherein said propyne substitution is to a thymidine analog.

 222. The method of claim 211, wherein each said modified nucleotide of one
25 or more of said clusters of at least one of said nucleotide regions of said probe and said second nucleic acid contains the same modification.

 223. The method of claim 222, wherein said modification consists of a 2'-O-

methyl substitution to the ribofuranosyl moiety.

224. The method of claim 210, wherein at least one of said probe and said second nucleic acid includes one or more conjugate molecules.

5

225. The method of claim 211, wherein at least one of said probe and said second nucleic acid includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said probe and said second nucleic acid at a site located within one or more of said clusters contained in at least one of said nucleotide regions of said probe and said second nucleic acid.

10

226. The method of claim 211, wherein said probe is an oligonucleotide consisting of from about 10 to about 100 nucleotide bases.

15

227. The method of claim 211, wherein said probe is an oligonucleotide consisting of from about 10 to about 15 nucleotide bases.

228. The method of claim 211, wherein said probe is an oligonucleotide consisting of from about 12 to about 15 nucleotide bases.

20

229. The method of claim 211, wherein said probe further includes a label.

230. The method of claim 229, wherein said label is selected from the group consisting of:

25

- a) a radioisotope,
- b) an enzyme,
- c) an enzyme cofactor,
- d) an enzyme substrate.

- e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- h) a fluorescent molecule,
- 5 i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- l) a nucleotide base sequence region that is unable to stably

hybridize to either said analyte or said second nucleic acid under said conditions.

10

231. The method of claim 230, wherein said label is a chemiluminescent molecule.

15

232. The method of claim 231, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

20

233. The method of claim 231, wherein said chemiluminescent molecule is an acridinium ester derivative.

25

234. The method of claim 229, wherein said label is joined to said probe at a site located within one of said clusters contained in said first nucleotide region of said probe.

235. The method of claim 211, wherein said analyte consists of RNA.

236. The method of claim 235, wherein said RNA is rRNA or tRNA.

237. The method of claim 235, wherein said sample further contains DNA.

238. The method of claim 211, wherein said analyte consists of DNA.

239. The method of either claim 211 or 229, wherein at least one of said analyte, said probe and said second nucleic acid is directly or indirectly immobilized by a solid support.

240. A method for detecting the presence or amount of an analyte comprising a first nucleic acid in a sample suspected of containing said analyte, which comprises the steps of:

- 10 a) contacting the following components:
 - i) said sample with a probe comprising a second nucleic acid, wherein said probe contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said analyte under selective hybridization conditions;
 - 15 ii) said analyte with a third nucleic acid, wherein said third nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under selective hybridization conditions; and
 - 20 iii) said third nucleic acid with a fourth nucleic acid, wherein said fourth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said third nucleic acid under selective hybridization conditions,

 wherein said third nucleic acid cannot stably hybridize to said fourth nucleic acid unless said third nucleic acid is stably hybridized with said analyte,

25 wherein said analyte is unable to stably hybridize to either said second nucleotide region of said third nucleic acid or said fourth nucleic acid under said conditions,

 wherein said probe is unable to stably hybridize any of said second nucleotide region of said analyte and said third and fourth nucleic acids under said conditions.

wherein said third nucleic acid is unable to stably hybridize to said first nucleotide region of said analyte under said conditions,

wherein said fourth nucleic acid is unable to stably hybridize to said first nucleotide region of said third nucleic acid under said conditions, and

5 wherein at least one of said nucleotide regions of said probe and said third and fourth nucleic acids includes one or more modified nucleotides;

 b) subjecting the components of step (a) to selective hybridization conditions, so that

10 if said first nucleotide region of said probe includes one or more of said modified nucleotides, then

 i) the hybridization binding affinity between said analyte and said probe is greater than the hybridization binding affinity between said analyte and an unmodified form of said probe, under identical hybridization conditions, and

15 ii) the hybridization rate between said analyte and said probe is greater than the hybridization rate between said analyte and an unmodified form of said probe, under identical hybridization conditions,

 if said first nucleotide region of said third nucleic acid includes one or more of said modified nucleotides, then

20 i) the hybridization binding affinity between said analyte and said third nucleic acid is greater than the hybridization binding affinity between said analyte and an unmodified form of said third nucleic acid, under identical hybridization conditions, and

25 ii) the hybridization rate between said analyte and said third nucleic acid is greater than the hybridization rate between said analyte and an unmodified form of said third nucleic acid, under identical hybridization conditions,

 if at least one of said second nucleotide region of said third nucleic acid and said first nucleotide region of said fourth nucleic acid includes one or more of said modified nucleotides, then

i) the hybridization binding affinity between said third and fourth nucleic acids is greater than the hybridization binding affinity between unmodified forms of said third and fourth nucleic acids, under identical hybridization conditions, and

5 ii) the hybridization rate between said third and fourth nucleic acids is greater than the hybridization rate between unmodified forms of said third and fourth nucleic acids, under identical hybridization conditions; and

c) detecting said labeled probe hybridized with said analyte, as an indication of the presence or amount of said analyte in said sample.

10 241. The method of claim 240, wherein at least one of said nucleotide regions of said probe and said third and fourth nucleic acids includes one or more clusters of at least about 4 modified nucleotides.

15 242. The method of claim 240, wherein at least one of said nucleotide regions of said probe and said third and fourth nucleic acids includes one or more clusters of at least about 6 modified nucleotides.

20 243. The method of claim 240, wherein at least one of said nucleotide regions of said probe and said third and fourth nucleic acids includes one or more clusters of at least about 8 modified nucleotides.

25 244. The method of claim 240, wherein substantially all of the nucleotides contained in at least one of said nucleotide regions of said probe and said third and fourth nucleic acids are modified.

245. The method of claim 241, wherein at least one of said modified nucleotides includes a modification selected from the group consisting of:

a) a modification to the nitrogenous base;

- b) a modification to the sugar moiety;
- c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage; and
- e) a modification to the internucleotide linkage.

5

246. The method of claim 241, wherein at least one of said modified nucleotides includes two modifications selected from the group consisting of:

- a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;
- c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage; and
- e) a modification to the internucleotide linkage.

10

247. The method of claim 241, wherein at least one of said modified nucleotides includes a 2'-modification to the ribofuranosyl moiety selected from the group consisting of:

- a) an alkyl substitution;
- b) an alkoxy substitution; and
- c) a halide substitution.

20

248. The method of claim 241, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

249. The method of claim 241, wherein at least one of said modified nucleotides includes a propyne substitution to the nitrogenous base.

25

250. The method of claim 249, wherein said propyne substitution is to a cytidine analog.

251. The method of claim 249, wherein said propyne substitution is to a thymidine analog.

5 252. The method of claim 241, wherein each said modified nucleotide of one or more of said clusters of at least one of said nucleotide regions of said probe and said third and fourth nucleic acids contains the same modification.

253. The method of claim 252, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

10

254. The method of claim 240, wherein at least one of said probe and said third nucleic acid includes one or more conjugate molecules.

255. The method of claim 241, wherein at least one of said probe and said third nucleic acid includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said probe and said third and fourth nucleic acids at a site located within one or more of said clusters contained in at least one of said nucleotide regions of said probe and said third and fourth nucleic acids.

15

256. The method of claim 241, wherein said probe is an oligonucleotide consisting of from about 10 to about 100 nucleotide bases.

20

257. The method of claim 241, wherein said probe is an oligonucleotide consisting of from about 10 to about 15 nucleotide bases.

25

258. The method of claim 241, wherein said probe is an oligonucleotide consisting of from about 12 to about 15 nucleotide bases.

259. The method of claim 241, wherein said probe further includes a label.

260. The method of claim 259, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b) an enzyme,
- c) an enzyme cofactor,
- d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- h) a fluorescent molecule,
- i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- l) a nucleotide base sequence region that is unable to stably hybridize to any of said analyte and said third and fourth nucleic acids under said conditions.

261. The method of claim 260, wherein said label is a chemiluminescent molecule.

262. The method of claim 261, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

263. The method of claim 261, wherein said chemiluminescent molecule is an acridinium ester derivative.

264. The method of claim 259, wherein said label is joined to said probe at a site located within one of said clusters contained in said first nucleotide region of said probe.

265. The method of claim 241, wherein said analyte consists of RNA.

266. The method of claim 265, wherein said RNA is rRNA or tRNA.

267. The method of claim 265, wherein said sample further contains DNA.

268. The method of claim 241, wherein said analyte consists of DNA.

269. The method of either claim 241 or 259, wherein said fourth nucleic acid is immobilized by a solid support.

270. A method for detecting the presence or amount of an analyte comprising a first nucleic acid in a sample suspected of containing said analyte, which comprises the steps of:

a) contacting the following components:

i) said sample with a second nucleic acid, wherein said second nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said analyte under selective hybridization conditions;

ii) said analyte with a probe comprising a third nucleic acid, wherein said probe contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under selective hybridization conditions; and

iii) said probe with said second nucleic acid, wherein said second nucleic acid contains a second nucleotide base sequence region able to stably

hybridize to a second nucleotide base sequence region of said probe under selective hybridization conditions,

wherein said probe cannot stably hybridize to said analyte and said second nucleic acid unless said second nucleic acid is stably hybridized with said analyte,

5 wherein said analyte is unable to stably hybridize to either said second nucleotide region of said probe or said second nucleotide region of said second nucleic acid under said conditions,

10 wherein said probe is unable to stably hybridize to either said first nucleotide region of said analyte or said first nucleotide region of said second nucleic acid under said conditions,

wherein said second nucleic acid is unable to stably hybridize to either said second nucleotide region of said analyte or said first nucleotide region of said probe under said conditions, and

15 wherein at least one of said nucleotide regions of said probe and said second nucleic acid includes one or more modified nucleotides;

b) subjecting the components of step a) to selective hybridization conditions, so that

if said first nucleotide region of said probe includes one or more of said modified nucleotides, then

20 i) the hybridization binding affinity between said analyte and said probe is greater than the hybridization binding affinity between said analyte and an unmodified form of said probe, under identical hybridization conditions, and

25 ii) the hybridization rate between said analyte and said probe is greater than the hybridization rate between said analyte and an unmodified form of said probe, under identical hybridization conditions,

if said first nucleotide region of said second nucleic acid includes one or more of said modified nucleotides, then

i) the hybridization binding affinity between said analyte

and said second nucleic acid is greater than the hybridization binding affinity between said analyte and an unmodified form of said second nucleic acid, under identical hybridization conditions, and

ii) the hybridization rate between said analyte and said second nucleic acid is greater than the hybridization rate between said analyte and an unmodified form of said second nucleic acid, under identical hybridization conditions, and

if at least one of said second nucleotide region of said probe and said second nucleotide region of said second nucleic acid includes one or more of said modified nucleotides, then

10 i) the hybridization binding affinity between said probe and said second nucleic acid is greater than the hybridization binding affinity between unmodified forms of said probe and said second nucleic acid, under identical hybridization conditions, and

ii) the hybridization rate between said probe and said second nucleic acid is greater than the hybridization rate between unmodified forms of said probe and said second nucleic acid, under identical hybridization conditions; and

c) detecting said labeled probe hybridized with said analyte as an indication of the presence or amount of said analyte in said sample.

20 271. The method of claim 270, wherein at least one of said nucleotide regions of said probe and said second nucleic acid includes one or more clusters of at least about 4 modified nucleotides.

272. The method of claim 270, wherein at least one of said nucleotide
25 regions of said probe and said second nucleic acid includes one or more clusters of at least
about 6 modified nucleotides.

273. The method of claim 270, wherein at least one of said nucleotide

regions of said probe and said second nucleic acid includes one or more clusters of at least about 8 modified nucleotides.

274. The method of claim 270, wherein substantially all of the nucleotides contained in at least one of said nucleotide regions of said probe and said second nucleic acid are modified.

275. The method of claim 271, wherein at least one of said modified nucleotides includes a modification selected from the group consisting of:

- a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;
- c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage; and
- e) a modification to the internucleotide linkage.

276. The method of claim 271, wherein at least one of said modified nucleotides includes two modifications selected from the group consisting of:

- a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;
- c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage; and
- e) a modification to the internucleotide linkage.

277. The method of claim 271, wherein at least one of said modified nucleotides includes a 2'-modification to the ribofuranosyl moiety selected from the group consisting of:

- a) an alkyl substitution;
- b) an alkoxy substitution; and

c) a halide substitution.

278. The method of claim 271, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

279. The method of claim 271, wherein at least one of said modified nucleotides includes a propyne substitution to the nitrogenous base.

280. The method of claim 279, wherein said propyne substitution is to a cytidine analog.

281. The method of claim 279, wherein said propyne substitution is to a thymidine analog.

282. The method of claim 271, wherein each said modified nucleotide of one or more of said clusters of at least one of said nucleotide regions of said probe and said second nucleic acid contains the same modification.

283. The method of claim 282, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

284. The method of claim 270, wherein at least one of said probe and said second nucleic acid includes one or more conjugate molecules.

285. The method of claim 271, wherein at least one of said probe and said second nucleic acid includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said probe and said second nucleic acid at a site located within one or more of said clusters contained in at least one of said

nucleotide regions of said probe and said second nucleic acid.

286. The method of claim 271, wherein said probe is an oligonucleotide consisting of from about 10 to about 100 nucleotide bases.

5

287. The method of claim 271, wherein said probe is an oligonucleotide consisting of from about 10 to about 15 nucleotide bases.

288. The method of claim 271, wherein said probe is an oligonucleotide consisting of from about 12 to about 15 nucleotide bases.

10

289. The method of claim 271, wherein said probe further includes a label.

290. The method of claim 289, wherein said label is selected from the group consisting of:

15

- a) a radioisotope,
- b) an enzyme,
- c) an enzyme cofactor,
- d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- h) a fluorescent molecule,
- i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- l) a nucleotide base sequence region that is unable to stably

20

25

hybridize to either said analyte or said second nucleic acid under said conditions.

291. The method of claim 290, wherein said label is a chemiluminescent molecule.

292. The method of claim 291, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

293. The method of claim 291, wherein said chemiluminescent molecule is an acridinium ester derivative.

294. The method of claim 289, wherein said label is joined to said probe at a site located within one of said clusters contained in said first nucleotide region of said probe.

295. The method of claim 271, wherein said analyte consists of RNA.

296. The method of claim 295, wherein said RNA is rRNA or tRNA.

297. The method of claim 295, wherein said sample further contains DNA.

298. The method of claim 271, wherein said analyte consists of DNA.

299. The method of claim either 271 or 289, wherein at least one of said analyte, said probe and said second nucleic acid is directly or indirectly immobilized by a solid support.

300. A method for detecting the presence or amount of an analyte comprising a first nucleic acid in a sample suspected of containing said analyte, which comprises the steps of:

a) contacting the following components:

5 i) said sample with a probe comprising a second nucleic acid, wherein said probe contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said analyte under selective hybridization conditions;

ii) said probe with a third nucleic acid, wherein said third nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said probe under selective hybridization conditions; and

10 iii) said third nucleic acid with a fourth nucleic acid, wherein said fourth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said third nucleic acid under selective hybridization conditions,

15 wherein said analyte is unable to stably hybridize to any of said second nucleotide region of said probe and said third and fourth nucleic acids under said conditions,

wherein said probe is unable to stably hybridize to either said second nucleotide region of said third nucleic acid or said fourth nucleic acid under said conditions,

wherein said third nucleic acid is unable to stably hybridize to said first nucleotide region of said probe under said conditions,

20 wherein said fourth nucleic acid is unable to stably hybridize to said first nucleotide region of said third nucleic acid under said conditions, and

wherein said first nucleotide region of said third nucleic acid is unable to stably hybridize to said fourth nucleic acid under said conditions;

25 b) subjecting the components of step a) to selective hybridization conditions; and

c) detecting said probe hybridized with said analyte as an indication of the presence or amount of said analyte in said sample.

301. The method of claim 300, wherein said probe further includes a label.

302. The method of claim 301, wherein said label is joined to said probe at a site located within said first nucleotide region of said probe.

303. The method of either claim 300 or 301, wherein said fourth nucleic acid is immobilized by a solid support.

304. A method for detecting the presence or amount of an analyte comprising a first nucleic acid in a sample suspected of containing said analyte, which comprises the steps of:

a) contacting the following components:

i) said sample with a probe comprising a second nucleic acid, wherein said probe contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said analyte under selective hybridization conditions;

ii) said analyte with a third nucleic acid, wherein said third nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under selective hybridization conditions; and

iii) said third nucleic acid with a fourth nucleic acid, wherein said fourth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said third nucleic acid under selective hybridization conditions,

wherein said analyte is unable to stably hybridize to either said second nucleotide region of said third nucleic acid or said fourth nucleic acid under said conditions,

wherein said probe is unable to stably hybridize to any of said second nucleotide region of said analyte and said third and fourth nucleic acids under said conditions.

and

wherein said first nucleotide region of said third nucleic acid is unable to stably hybridize to said fourth nucleic acid under said conditions;

b) subjecting the components of step a) to selective hybridization conditions; and

c) detecting said probe hybridized with said analyte as an indication of the presence or amount of said analyte in said sample.

305. The method of claim 304, wherein said probe further includes a label.

306. The method of claim 305, wherein said label is joined to said probe at a site located within said first nucleotide region of said probe.

307. The method of either claim 304 or 305, wherein said fourth nucleic acid is immobilized by a solid support.

308. A method for detecting the presence or amount of an analyte comprising a first nucleic acid in a sample suspected of containing said analyte, which comprises the steps of:

- a) contacting the following components:
 - i) said sample with a probe comprising a second nucleic acid, wherein said probe contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said analyte under selective hybridization conditions;
 - ii) said analyte with a third nucleic acid, wherein said third nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under selective hybridization conditions;

iii) said probe with said third nucleic acid, wherein said third nucleic acid contains a second nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said probe under selective hybridization conditions; and

5 iv) said third nucleic acid with a fourth nucleic acid, wherein said fourth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a third nucleotide base sequence region of said third nucleic acid under selective hybridization conditions,

10 wherein said analyte is unable to stably hybridize to any of said second nucleotide region of said probe, said second and third nucleotide regions of said third nucleic acid, and said fourth nucleic acid under said conditions,

wherein said probe is unable to stably hybridize to any of said second nucleotide region of said analyte, said first and third nucleotide regions of said third nucleic acid, and said fourth nucleic acid under said conditions, and

15 wherein said first and second nucleotide regions of said third nucleic acid do not stably hybridize to said fourth nucleic acid under said conditions;

b) subjecting the components of step a) to selective hybridization conditions; and

20 c) detecting said probe hybridized with said analyte as an indication of the presence or amount of said analyte in said sample.

309. The method of claim 308, wherein said probe further includes a label.

25 310. The method of claim 309, wherein said label is joined to said probe at a site located within said first nucleotide region of said probe.

311. The method of either claim 308 or 309, wherein said fourth nucleic acid is immobilized by a solid support.

312. A method for detecting the presence or amount of an analyte comprising a first nucleic acid in a sample suspected of containing said sample, which comprises the steps of:

a) contacting the following components:

5 i) said sample with a probe comprising a second nucleic acid, wherein said probe contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said analyte under selective hybridization conditions;

10 ii) said probe with a first coupling nucleic acid comprising a third nucleic acid, wherein said first coupling nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said probe under selective hybridization conditions;

15 iii) a second coupling nucleic acid comprising a fourth nucleic acid with a fifth nucleic acid, wherein said fifth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said second coupling nucleic acid under selective hybridization conditions; and, alternatively,

20 (a) said first coupling nucleic acid with said second coupling nucleic acid, wherein said second coupling nucleic acid contains a second nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said first coupling nucleic acid under selective hybridization conditions; or

25 (b) one or more optional coupling nucleic acids, other than said first and second coupling nucleic acids, wherein each of said optional coupling nucleic acids is able to stably hybridize to at least two other coupling nucleic acids under selective hybridization conditions, wherein at least one of said other coupling nucleic acids may be at least one of said first and second coupling nucleic acids, and wherein each of said other coupling nucleic acids is directly or indirectly joined to said probe and said fifth nucleic acid under selective hybridization conditions.

wherein said analyte is unable to stably hybridize to any of said second nucleotide region of said probe, said first and second coupling nucleic acids, said fifth nucleic acid, and any of said optional coupling nucleic acids under said conditions.

5 wherein said probe is unable to stably hybridize to any of said second nucleotide region of said first coupling nucleic acid, said second coupling nucleic acid, said fifth nucleic acid, and any of said optional coupling nucleic acids under said conditions.

wherein said fifth nucleic acid is unable to stably hybridize to any of said first coupling nucleic acid, said second nucleotide region of said second coupling nucleic acid, and any of said optional coupling nucleic acids under said conditions

10 wherein said first coupling nucleic acid is unable to stably hybridize to said first nucleotide region of said second coupling nucleic acid under said conditions, and

wherein said second coupling nucleic acid is unable to stably hybridize to said first nucleotide region of said first coupling nucleic acid under said conditions;

15 b) subjecting the components of step a) to selective hybridization conditions; and

c) detecting said probe hybridized with said analyte as an indication of the presence or amount of said analyte in said sample.

20 313. The method of claim 312, wherein said probe includes a label.

314. The method of claim 313, wherein said label is joined to said probe at a site located within said first nucleotide region of said probe.

25 315. The method of either claim 312 or 313, wherein said fifth nucleic acid is immobilized by a solid support.

316. A method for detecting the presence or amount of an analyte comprising a first nucleic acid in a sample suspected of containing said sample, which

comprises the steps of:

a) contacting the following components:

i) said sample with a probe comprising a second nucleic acid, wherein said probe contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said analyte under selective hybridization conditions;

ii) said analyte with a first coupling nucleic acid comprising a third nucleic acid, wherein said first coupling nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under selective hybridization conditions;

iii) a second coupling nucleic acid comprising a fourth nucleic acid with a fifth nucleic acid, wherein said fifth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said second coupling nucleic acid under selective hybridization conditions; and, alternatively,

(a) said first coupling nucleic acid with said second coupling nucleic acid, wherein said second coupling nucleic acid contains a second nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said first coupling nucleic acid under selective hybridization conditions; or

(b) one or more optional coupling nucleic acids, other than said first and second coupling nucleic acids, wherein each of said optional coupling nucleic acids is able to stably hybridize to at least two other coupling nucleic acids under selective hybridization conditions, wherein at least one of said other coupling nucleic acids may be at least one of said first and second coupling nucleic acids, and wherein each of said other coupling nucleic acids is directly or indirectly joined to said probe and said fifth nucleic acid under selective hybridization conditions,

wherein said analyte is unable to stably hybridize to any of said second nucleotide region of said first coupling nucleic acid, said second coupling nucleic acid, said

fifth nucleic acid, and any of said optional coupling nucleic acids under said conditions,
wherein said probe is unable to stably hybridize to any of said second
nucleotide region of said analyte, said first and second coupling nucleic acids, said fifth
nucleic acid, and any of said optional coupling nucleic acids under said conditions,

5 wherein said fifth nucleic acid is unable to stably hybridize to any of said first
coupling nucleic acid, said second nucleotide region of said second coupling nucleic acid, and
any of said optional coupling nucleic acids under said conditions,

wherein said first coupling nucleic acid is unable to stably hybridize to said
first nucleotide region of said second coupling nucleic acid under said conditions, and

10 wherein said second coupling nucleic acid is unable to stably hybridize to said
first nucleotide region of said first coupling nucleic acid under said conditions;

b) subjecting the components of step a) to selective hybridization
conditions; and

c) detecting said probe hybridized with said analyte as an indication
15 of the presence or amount of said analyte in said sample.

317. The method of claim 316, wherein said probe further includes a label.

20 318. The method of claim 317, wherein said label is joined to said probe at a
site located within said first nucleotide region of said probe.

319. The method of either claim 316 or 317, wherein said fifth nucleic acid
is immobilized by a solid support.

25 320. A method for detecting the presence or amount of an analyte
comprising a first nucleic acid in a sample suspected of containing said sample, which
comprises the steps of:

a) contacting the following components:

i) said sample with a probe comprising a second nucleic acid, wherein said probe contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said analyte under selective hybridization conditions;

5 ii) said probe with a first coupling nucleic acid comprising a third nucleic acid, wherein said first coupling nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said probe under selective hybridization conditions;

10 iii) said analyte with said first coupling nucleic acid, wherein said first coupling nucleic acid contains a second nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under selective hybridization conditions;

15 iv) a second coupling nucleic acid comprising a fourth nucleic acid with a fifth nucleic acid, wherein said fifth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said second coupling nucleic acid under selective hybridization conditions; and, alternatively,

20 (a) said first coupling nucleic acid with said second coupling nucleic acid, wherein said second coupling nucleic acid contains a second nucleotide base sequence region able to stably hybridize to a third nucleotide base sequence region of said first coupling nucleic acid under selective hybridization conditions; or

25 (b) one or more optional coupling nucleic acids, other than said first and second coupling nucleic acids, wherein each of said optional coupling nucleic acids is able to stably hybridize to at least two other coupling nucleic acids under selective hybridization conditions, wherein at least one of said other coupling nucleic acids may be at least one of said first and second coupling nucleic acids, and wherein each of said other coupling nucleic acids is directly or indirectly joined to said analyte, said probe and said fifth nucleic acid under selective hybridization conditions.

wherein said analyte is unable to stably hybridize to any of said second nucleotide region of said probe, said first and third nucleotide regions of said first coupling nucleic acid, said second coupling nucleic acid, said fifth nucleic acid, and any of said optional coupling nucleic acids under said conditions.

5 wherein said probe is unable to stably hybridize to any of said second nucleotide region of said analyte, said second and third nucleotide regions of said first coupling nucleic acid, said second coupling nucleic acid, said fifth nucleic acid, and any of said optional coupling nucleic acids under said conditions,

10 wherein said fifth nucleic acid is unable to stably hybridize to any of said first coupling nucleic acid, said second nucleotide region of said second coupling nucleic acid, and any of said optional coupling nucleic acids under said conditions,

wherein said first coupling nucleic acid is unable to stably hybridize to said second nucleotide region of said second coupling nucleic acid, and

15 wherein said second coupling nucleic acid is unable to stably hybridize to either said first or second nucleotide region of said first coupling nucleic acid;

b) subjecting the components of step a) to selective hybridization conditions; and

c) detecting said probe hybridized with said analyte as an indication of the presence or amount of said analyte in said sample.

20

321. The method of claim 320, wherein said probe further includes a label.

322. The method of claim 321, wherein said label is joined to said probe at a site located within said first nucleotide region of said probe.

25

323. The method of either claim 320 or 321, wherein said fifth nucleic acid is immobilized by a solid support.

324. A method of amplifying a target comprising a first nucleic acid, which comprises the steps of:

a) contacting a sample suspected of containing said target with a second nucleic acid, wherein said second nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said target under amplification conditions, and wherein said first nucleotide region of said second nucleic acid includes one or more modified nucleotides;

b) subjecting the components of step a) to said amplification conditions, so that

i) the hybridization binding affinity between said target and said second nucleic acid is greater than the hybridization binding affinity between said target and an unmodified form of said second nucleic acid, and

ii) the hybridization rate between said target and said second nucleic acid is greater than the hybridization rate between said target and an unmodified form of said second nucleic acid; and

c) incubating the components of step a) under said amplification conditions, such that said target is amplified.

325. The method of claim 324, wherein said first nucleotide region of said second nucleic acid includes one or more clusters of at least about 4 modified nucleotides.

326. The method of claim 324, wherein said first nucleotide region of said second nucleic acid includes one or more clusters of at least about 6 modified nucleotides.

327. The method of claim 324, wherein said first nucleotide region of said second nucleic acid includes one or more clusters of at least about 8 modified nucleotides.

328. The method of claim 324, wherein substantially all of the nucleotides

contained in said first nucleotide region of said second nucleic acid are modified.

329. The method of claim 325, wherein at least one of said modified nucleotides includes a modification selected from the group consisting of:

- a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;
- c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage; and
- e) a modification to the internucleotide linkage.

330. The method of claim 325, wherein at least one of said modified nucleotides includes two modifications selected from the group consisting of:

- a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;
- c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage; and
- e) a modification to the internucleotide linkage.

331. The method of claim 325, wherein at least one of said modified nucleotides includes a 2'-modification to the ribofuranosyl moiety selected from the group consisting of:

- a) an alkyl substitution;
- b) an alkoxy substitution; and
- c) a halide substitution.

332. The method of claim 325, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

333. The method of claim 325, wherein at least one of said modified nucleotides includes a propyne substitution to the nitrogenous base.

5 334. The method of claim 333, wherein said propyne substitution is to a cytidine analog.

335. The method of claim 333, wherein said propyne substitution is to a thymidine analog.

10 336. The method of claim 325, wherein each said modified nucleotide of one or more of said clusters of said first nucleotide region of said second nucleic acid contains the same modification.

15 337. The method of claim 336, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

338. The method of claim 324, wherein said second nucleic acid includes one or more conjugate molecules.

20 339. The method of claim 325, wherein said second nucleic acid includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to said second nucleic acid at a site located within one or more of said clusters contained in said first nucleotide region of said second nucleic acid.

25 340. The method of claim 325, wherein said contacting step further includes contacting said second nucleic acid with nucleotide triphosphates and at least one nucleic acid polymerase.

341. The method of claim 340, wherein said second nucleic acid is capable of having nucleotide bases added to its 3' terminus by at least one of said polymerases.

342. The method of claim 325 or 341, wherein said contacting step further
5 includes contacting said target with a third nucleic acid, wherein said third nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said target under selective hybridization conditions,

wherein said second nucleic acid is unable to stably hybridize to either said
10 second nucleotide region of said target or said third nucleic acid under said hybridization conditions, and

wherein said third nucleic acid is unable to stably hybridize to said first
nucleotide region of said target under said conditions.

343. The method of claim 342, wherein the formation of a stable hybrid
15 between said third nucleic acid and said target under said conditions reduces or prevents amplification of said target.

344. The method of claim 342, wherein said first and second nucleotide
20 regions of said target constitute substantially the same nucleotide region.

345. The method of claim 342, wherein said first nucleotide region of said
third nucleic acid includes one or more modified nucleotides.

346. The method of claim 342, wherein said first nucleotide region of said
25 third nucleic acid includes one or more clusters of at least about 4 modified nucleotides.

347. The method of claim 342, wherein substantially all of the nucleotides
contained in said first nucleotide region of said third nucleic acid are modified.

348. The method of claim 347, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

349. The method of claim 347, wherein each said modified nucleotide of one or more of said clusters contains the same modification.

350. The method of claim 349, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

351. The method of claim 342, wherein at least one of said second and third nucleic acids includes one or more *conjugate molecules*.

352. The method of claim 346, wherein at least one of said second and third nucleic acids includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said second and third nucleic acids at a site located within one or more of said clusters contained in at least one of said first nucleotide region of said second nucleic acid and said first nucleotide region of said third nucleic acid.

353. The method of claim 342, wherein said second nucleic acid contains a 5' nucleotide base sequence region which is unable to stably hybridize to said target under said conditions.

354. The method of claim 353, wherein any of said target and said second and third nucleic acids is directly or indirectly immobilized by a solid support.

355. A kit comprising a nucleic acid probe for use in detecting the presence or amount of a nucleic acid analyte in a sample suspected of containing said analyte, wherein said probe comprises a first nucleotide base sequence region able to stably hybridize to a first

nucleotide base sequence region of said analyte under selective hybridization conditions, and wherein said first nucleotide region of said probe includes at least about 4 modified nucleotide bases, so that

5 a) the hybridization binding affinity between said analyte and said probe is greater than the hybridization binding affinity between said analyte and an unmodified form of said probe, under said conditions, and

b) the hybridization rate between said analyte and said probe is greater than the hybridization rate between said analyte and an unmodified form of said probe, under said conditions.

10

356. The kit of claim 355, wherein said first nucleotide region of said probe includes one or more clusters of at least about 6 modified nucleotides.

15

357. The kit of claim 355, wherein said first nucleotide region of said probe includes one or more clusters of at least about 8 modified nucleotides.

358. The kit of claim 355, wherein substantially all of the nucleotides contained in said first nucleotide region of said probe are modified.

20

359. The kit of claim 355, wherein at least one of said modified nucleotide bases contains a modification selected from the group consisting of:

25

- a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;
- c) a modification to phosphate moiety;
- d) a modification to the internucleoside linkage; and
- e) a modification to the internucleotide linkage moiety.

360. The kit of claim 355, wherein at least one of said modified nucleotides

includes two modifications selected from the group consisting of:

- a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;
- c) a modification to phosphate moiety;
- d) a modification to the internucleoside linkage; and
- e) a modification to the internucleotide linkage moiety.

361. The kit of claim 355, wherein at least one of said modified nucleotides contains a 2'-modification to the ribofuranosyl moiety selected from the group consisting of:

- a) an alkyl substitution;
- b) an alkoxy substitution; and
- c) a halide substitution.

362. The kit of claim 355, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

363. The kit of claim 355, wherein at least one of said modified nucleotides includes a propyne substitution to the nitrogenous base.

364. The kit of claim 363, wherein said propyne substitution is to a cytidine analog.

365. The kit of claim 363, wherein said propyne substitution is to a thymidine analog.

366. The kit of claim 355, wherein said probe includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to said probe at a site located within one or more of said clusters contained in said first nucleotide

region of said probe.

367. The kit of claim 355, wherein said probe consists of from about 10 to about 100 nucleotides.

368. The kit of claim 355, wherein said probe consists of from about 10 to about 15 nucleotides.

369. The kit of claim 355, wherein said probe consists of from about 12 to about 15 nucleotides.

370. The kit of claim 355, wherein said probe further includes a label.

371. The kit of claim 370, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b) an enzyme,
- c) an enzyme cofactor,
- d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- h) a fluorescent chemiluminescent molecule,
- i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- l) a base sequence region that is unable to stably hybridize to said analyte under said conditions.

372. The kit of claim 371, wherein said label is a chemiluminescent molecule.

373. The kit of claim 372, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

374. The kit of claim 372, wherein said label is an acridinium ester derivative.

375. The kit of either claim 355 or 370, further comprising a solid support.

376. The kit of claim 375, wherein said probe is directly or indirectly immobilized by said solid support.

377. The kit of claim 355 or 370, further comprising one or more nucleic acid controls.

378. The kit of claim 355 or 370, further comprising one or more hybridization reagents.

379. A kit comprising a first nucleic acid capable of amplifying an analyte comprising a second nucleic acid in a sample suspected of containing said analyte, wherein said first nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under amplification conditions, and wherein said first nucleotide region of said first nucleic acid includes at least about 4 modified nucleotides, so that

a) the hybridization binding affinity between said analyte and said first nucleic acid is greater than the hybridization binding affinity between said analyte and an

unmodified form of said first nucleic acid, under said conditions, and

b) the hybridization rate between said analyte and said first nucleic acid is greater than the hybridization rate between said analyte and an unmodified form of said first nucleic acid, under said conditions.

5

380. The kit of claim 379, wherein said first nucleotide region of said first nucleic acid includes one or more clusters of at least about 6 modified nucleotides.

10

381. The kit of claim 379, wherein said first nucleotide region of said first nucleic acid includes one or more clusters of at least about 8 modified nucleotides.

382. The kit of claim 379, wherein substantially all of the nucleotides contained in said first nucleotide region of said first nucleic acid are modified.

15

383. The kit of claim 379, wherein at least one of said modified nucleotide bases contains a modification selected from the group consisting of:

20

- a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;
- c) a modification to phosphate moiety;
- d) a modification to the internucleoside linkage; and
- e) a modification to the internucleotide linkage moiety.

25

384. The kit of claim 379, wherein at least one of said modified nucleotides includes two modifications selected from the group consisting of:

- a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;
- c) a modification to phosphate moiety;
- d) a modification to the internucleoside linkage; and

- e) a modification to the internucleotide linkage moiety.

385. The kit of claim 379, wherein at least one of said modified nucleotides contains a 2'-modification to the ribofuranosyl moiety selected from the group consisting of:

- 5 a) an alkyl substitution;
 b) an alkoxy substitution; and
 c) a halide substitution.

10 386. The kit of claim 379, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

387. The kit of claim 379, wherein at least one of said modified nucleotides includes a propyne substitution to the nitrogenous base.

15 388. The kit of claim 387, wherein said propyne substitution is to a cytidine analog.

389. The kit of claim 387, wherein said propyne substitution is to a thymidine analog.

20 390. The kit of claim 379, wherein said first nucleic acid includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to said first nucleic acid at a site located within one or more of said clusters contained in said first nucleotide region of said first nucleic acid.

25 391. The kit of claim 379, further comprising a solid support.

392. The kit of claim 391, wherein said first nucleic acid is directly or

indirectly immobilized by said solid support.

393. The kit of claim 392, further comprising nucleotide triphosphates and at least one nucleic acid polymerase.

5

394. The kit of claim 393, wherein said first nucleic acid is capable of having nucleotide bases added to its 3' terminus by at least one of said nucleic acid polymerases.

10

395. The kit of claim 379, further comprising one or more amplification reagents.

15

396. The kit of claim 379, further comprising a third nucleic acid, wherein said third nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte, such that said analyte is able to stably hybridize to said third nucleic acid under selective hybridization conditions,

wherein said first oligonucleotide is unable to stably hybridize to either said second nucleotide region of said analyte or said third nucleic acid under said hybridization conditions, and

20

wherein said third nucleic acid is unable to stably hybridize to said first nucleotide region of said analyte under said conditions.

25

397. The kit of claim 396, wherein the formation of a stable hybrid between said third nucleic acid and said analyte under said conditions reduces or prevents amplification of said analyte.

398. The kit of claim 396, wherein said first and second nucleotide regions of said analyte constitute substantially the same nucleotide region.

399. The kit of claim 396, further comprising one or more hybridization reagents.

5 400. A nucleic acid probe comprising a nucleotide base sequence region containing one or more clusters of at least about 4 modified nucleotides, wherein said probe includes at least one label joined to said probe at a site located within one of said clusters contained in said nucleotide region.

10 401. The probe of claim 400, wherein said nucleotide region of said probe includes one or more clusters of at least about 6 modified nucleotides.

402. The probe of claim 400, wherein said nucleotide region of said probe includes one or more clusters of at least about 8 modified nucleotides.

15 403. The probe of claim 400, wherein substantially all of the nucleotides contained in said nucleotide region of said probe are modified.

404. The probe of claim 400, wherein at least one of said modified nucleotides includes a modification selected from the group consisting of:

- 20
- a) a modification to the nitrogenous base;
 - b) a modification to the sugar moiety;
 - c) a modification to the phosphate moiety;
 - d) a modification to the internucleoside linkage; and
 - e) a modification to the internucleotide linkage.

25 405. The probe of claim 400, wherein at least one of said modified nucleotides includes two modifications selected from the group consisting of:

- a) a modification to the nitrogenous base;

- b) a modification to the sugar moiety;
- c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage; and
- e) a modification to the internucleotide linkage.

5

406. The probe of claim 400, wherein at least one of said modified nucleotides includes a 2'-modification to the ribofuranosyl moiety selected from the group consisting of:

10

- a) an alkyl substitution;
- b) an alkoxy substitution; and
- c) a halide substitution.

15

407. The probe of claim 400, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

20

408. The probe of claim 400, wherein at least one of said modified nucleotides includes a propyne substitution to the nitrogenous base.

409. The probe of claim 408, wherein said propyne substitution is to a cytidine analog.

410. The probe of claim 408, wherein said propyne substitution is to a thymidine analog.

25

411. The probe of claim 400, wherein each said modified nucleotide of one or more of said clusters contains the same modification.

412. The probe of claim 411, wherein said modification consists of a 2'-O-

methyl substitution to the ribofuranosyl moiety.

5 413. The probe of claim 400, wherein said probe includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to said probe at a site located within one or more of said clusters contained in said nucleotide region of said probe.

10 414. The probe of claim 400, wherein said probe is an oligonucleotide consisting of from about 10 to about 100 nucleotide bases.

415. The probe of claim 400, wherein said probe is an oligonucleotide consisting of from about 10 to about 15 nucleotide bases.

15 416. The probe of claim 400, wherein said probe is an oligonucleotide consisting of from about 12 to about 15 nucleotide bases.

417. The probe of claim 400, wherein said probe further includes a label.

20 418. The probe of claim 417, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b) an enzyme,
- c) an enzyme cofactor,
- d) an enzyme substrate,
- 25 e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- h) a fluorescent molecule,

- i) a phosphorescent molecule,
 - j) an electrochemiluminescent molecule,
 - k) a chromophore, and
 - l) a nucleotide base sequence region that is unable to stably
- 5 hybridize to said analyte under said conditions.

419. The probe of claim 418, wherein said label is a chemiluminescent molecule.

10 420. The probe of claim 419, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

15 421. The probe of claim 419, wherein said chemiluminescent molecule is an acridinium ester derivative.

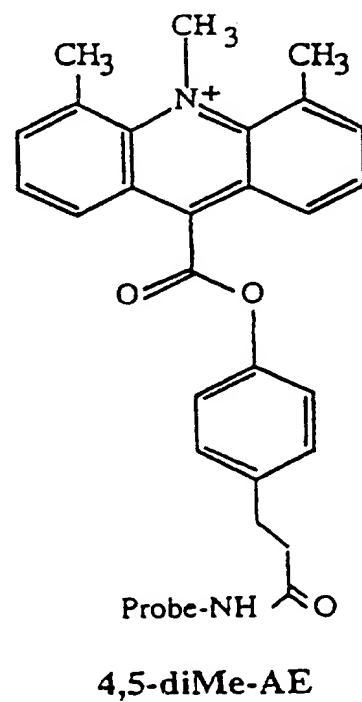
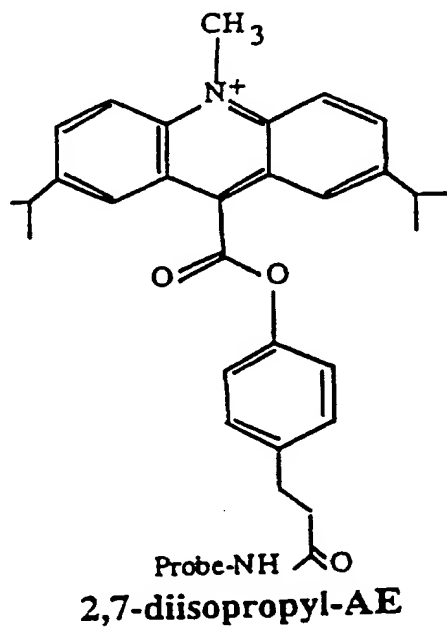
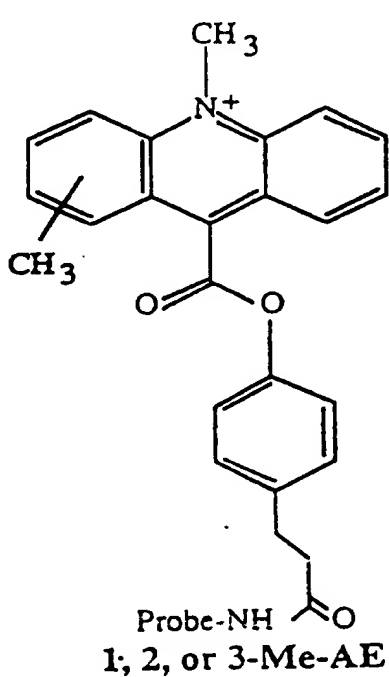
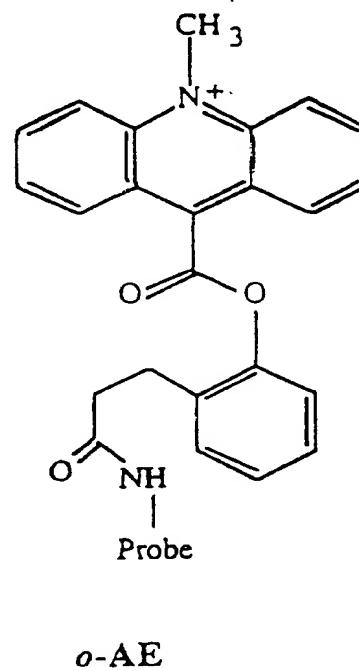
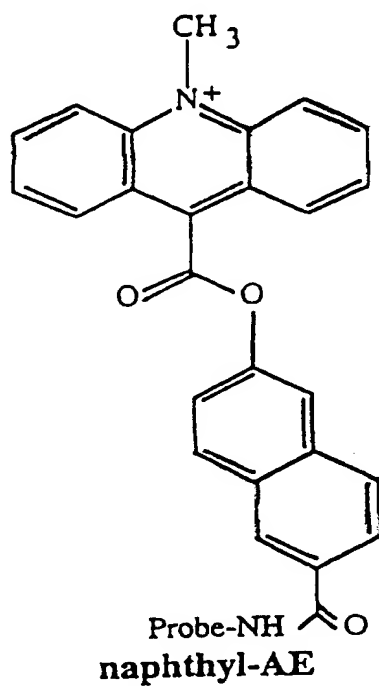
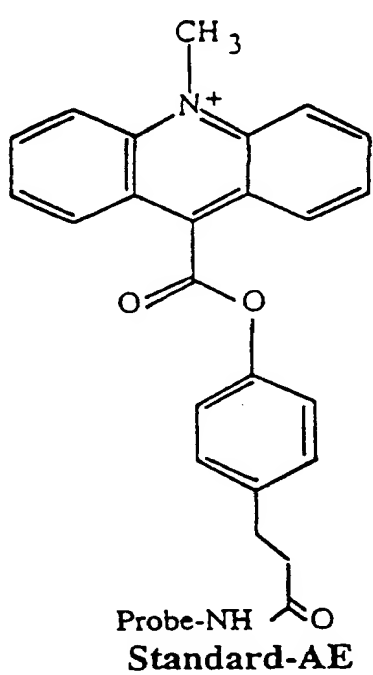


FIGURE 1A

2/9

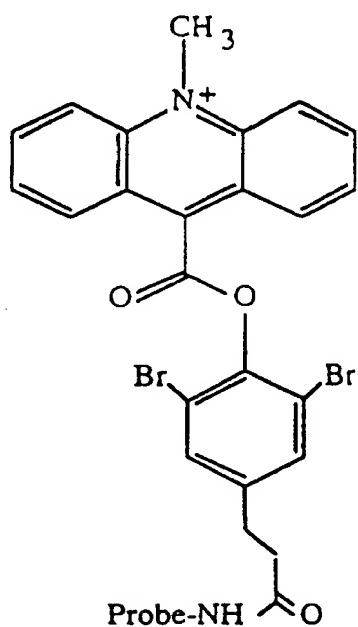
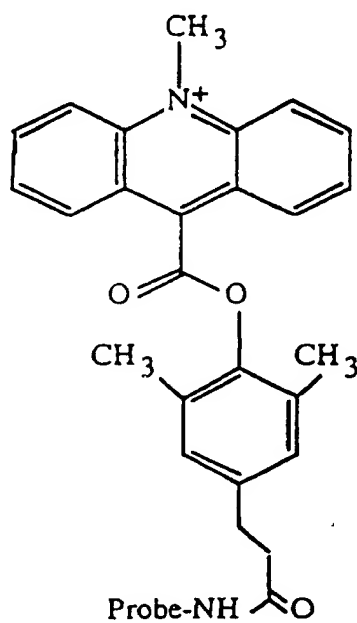
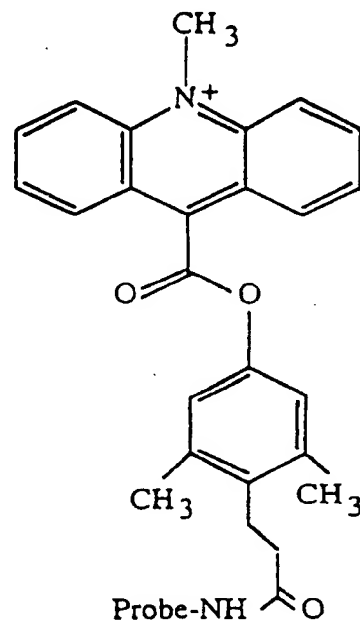
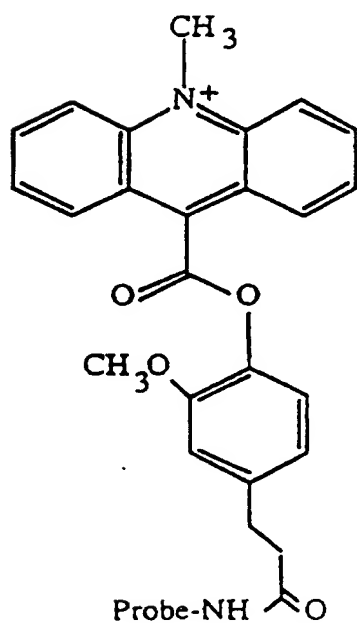
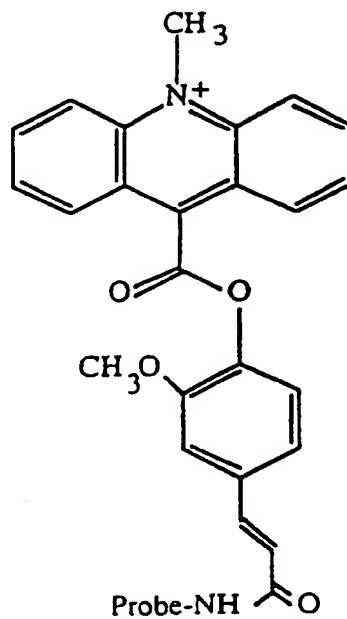
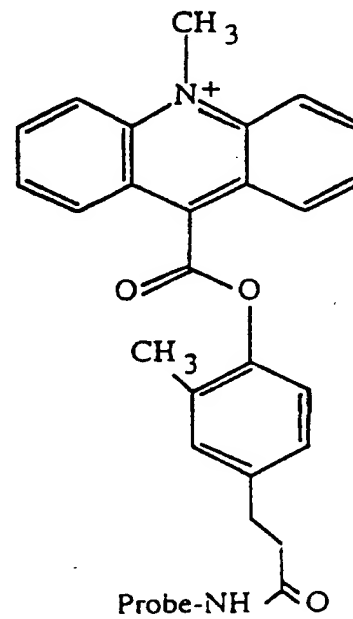
*o*-diBr-AE*o*-diMe-AE*m*-diMe-AE*o*-MeO-AE*o*-MeO (cinnamyl)-AE*o*-Me-AE

FIGURE 1B

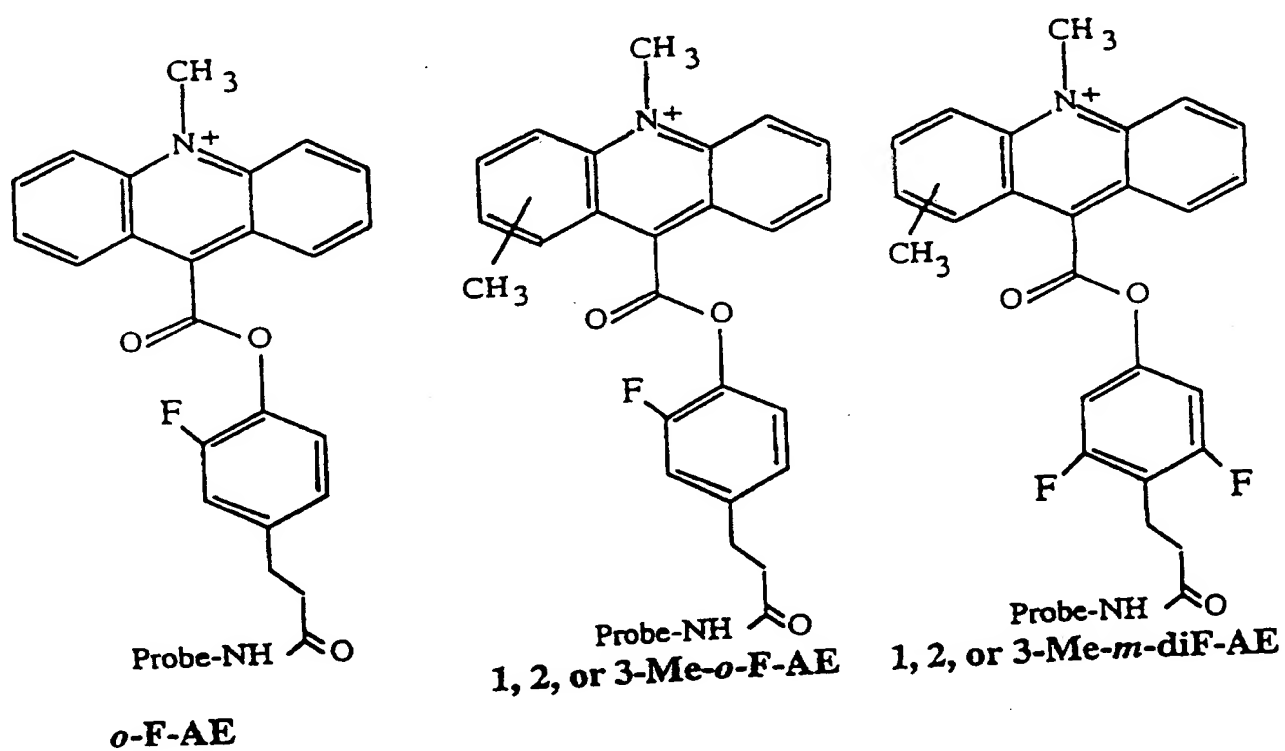


FIGURE 1C

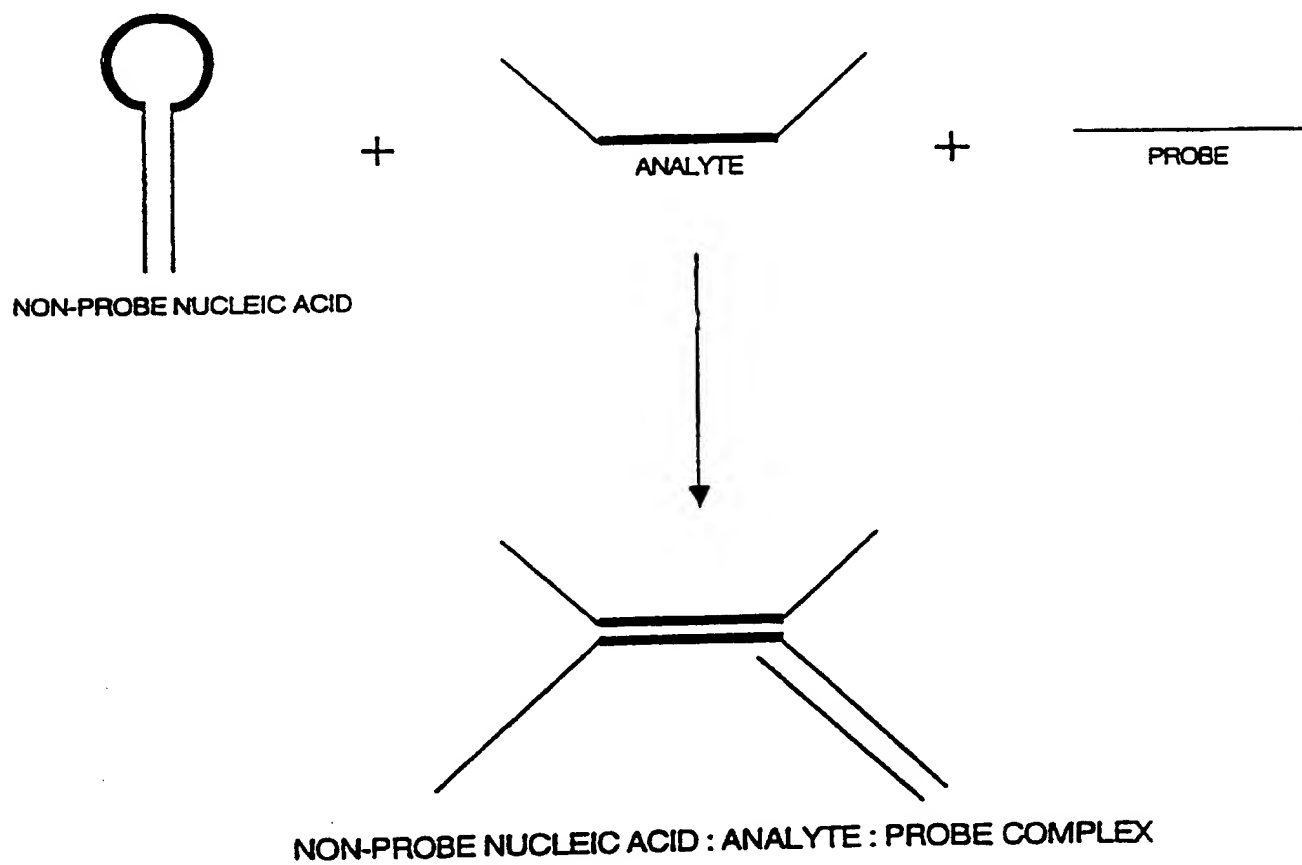


FIGURE 2

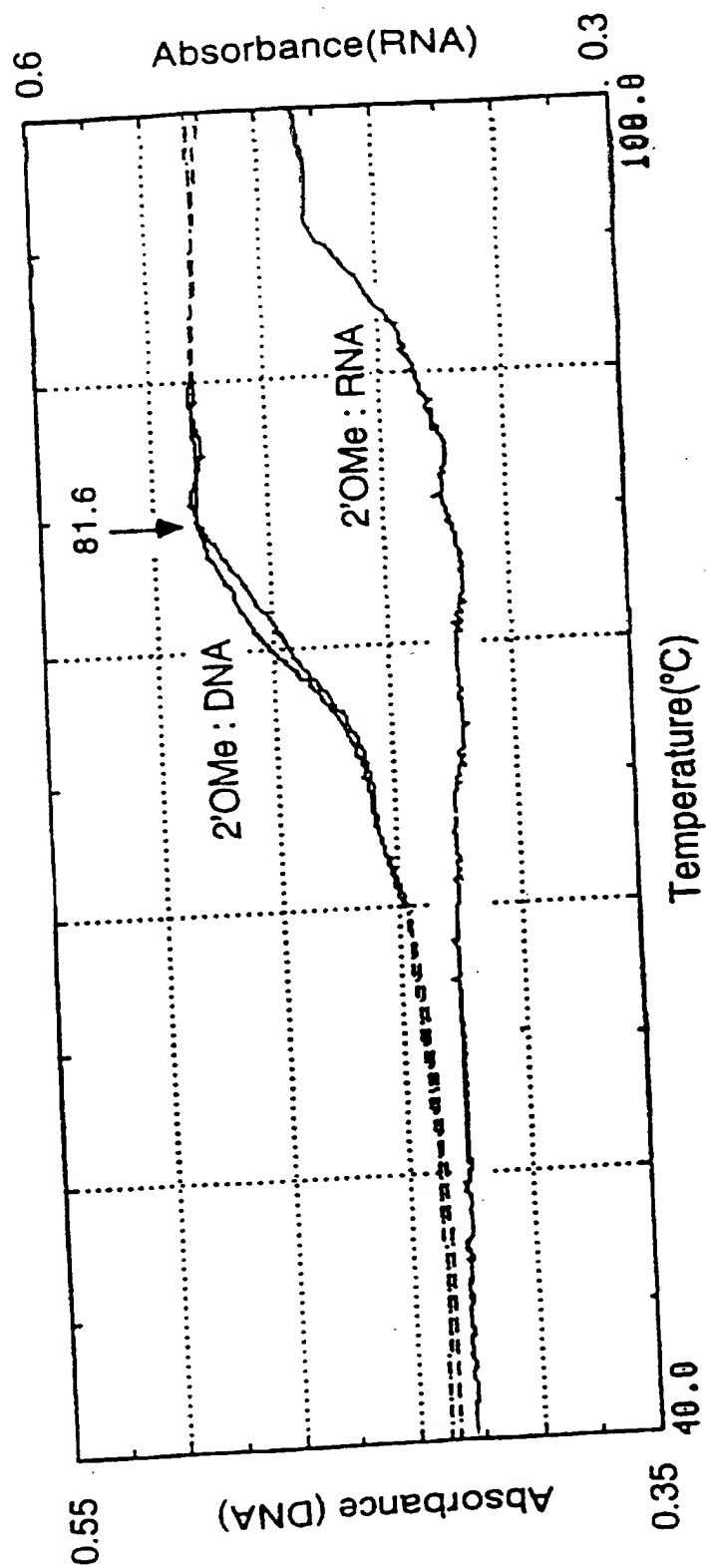


FIGURE 3

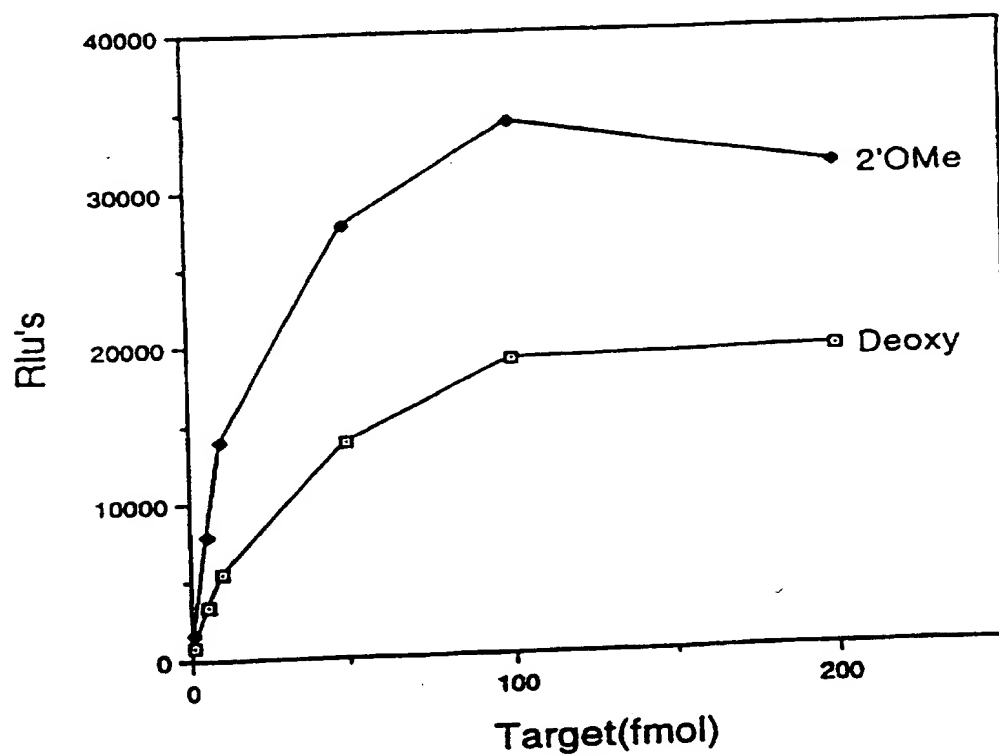


FIGURE 4

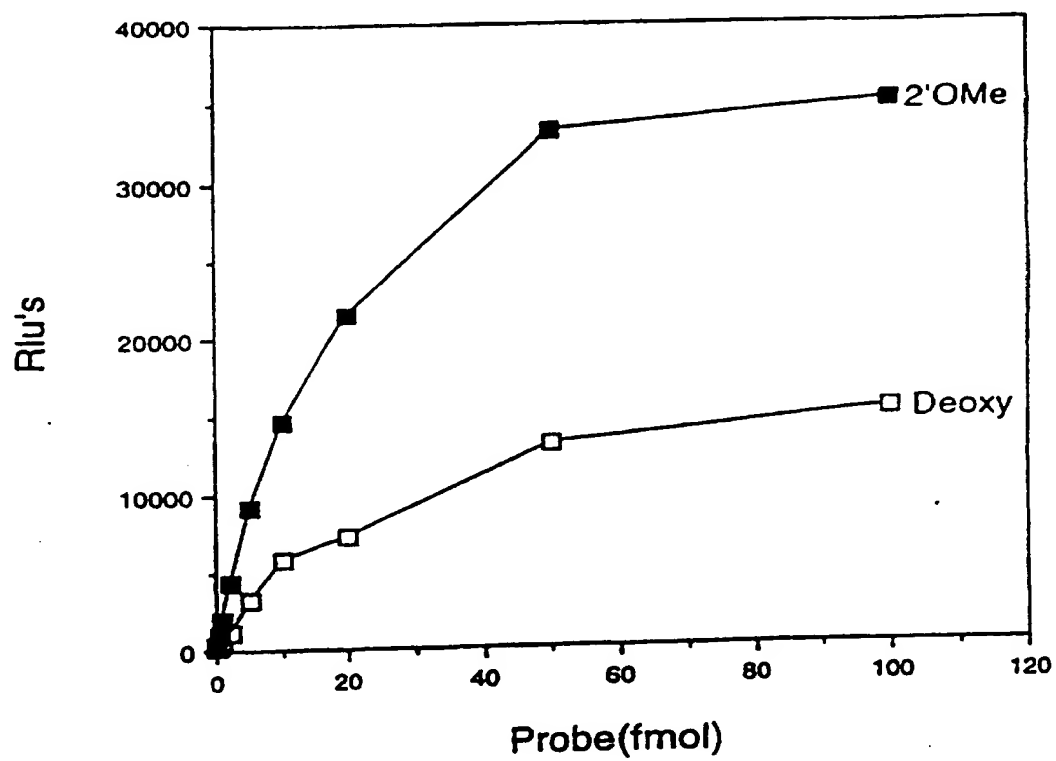


FIGURE 5

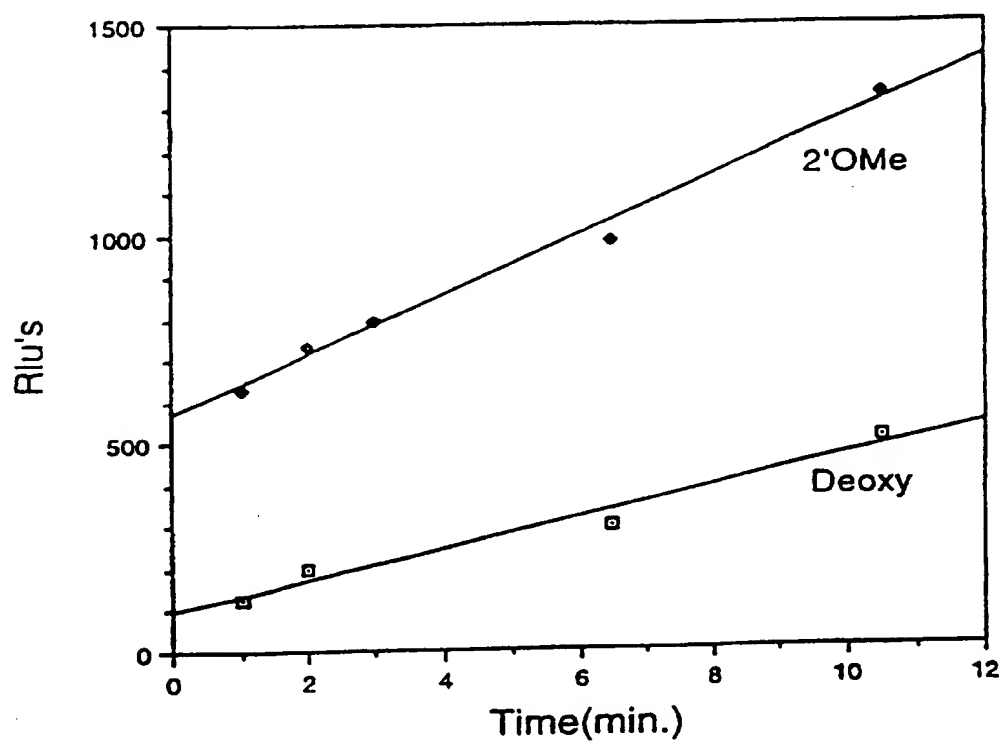


FIGURE 6

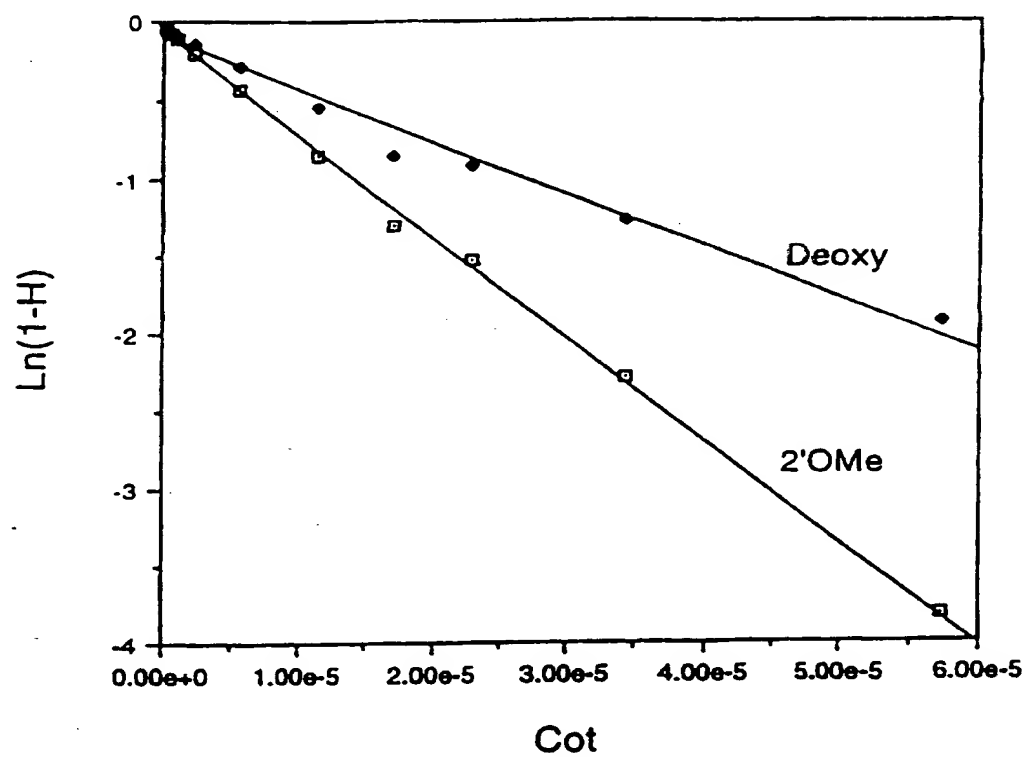


FIGURE 7

**CORRECTED
VERSION***

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/68	A2	(11) International Publication Number: WO 98/02582 (43) International Publication Date: 22 January 1998 (22.01.98)
(21) International Application Number: PCT/US97/12347 (22) International Filing Date: 15 July 1997 (15.07.97) (30) Priority Data: 60/021,818 16 July 1996 (16.07.96) US (71) Applicant: GEN-PROBE INCORPORATED [US/US]; 10210 Genetic Center Drive, San Diego, CA 92121 (US). (72) Inventors: BECKER, Michael, McClellan; 12819 Meadowdale Lane, San Diego, CA 92131 (US). MAJLESSI, Mehrdad; 15895 Sunnyfield Place, San Diego, CA 92127 (US). (74) Agents: HEBER, Sheldon, O. et al.; Lyon & Lyon LLP, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).		(81) Designated States: AU, CA, JP, KR, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: METHODS FOR DETECTING AND AMPLIFYING NUCLEIC ACID SEQUENCES USING MODIFIED OLIGONUCLEOTIDES HAVING INCREASED TARGET SPECIFIC T _M		
(57) Abstract <p>The present invention concerns oligonucleotides containing one or more modified nucleotides which increase the binding affinity of the oligonucleotides to target nucleic acids having a complementary nucleotide base sequence. These modified oligonucleotides hybridize to the target sequence at a faster rate than unmodified oligonucleotides having an identical nucleotide base sequence. Such modified oligonucleotides include oligonucleotides containing at least one 2'-O-methylribofuranosyl moiety joined to a nitrogenous base. Oligonucleotides can be modified in accordance with the present invention to preferentially bind RNA targets. The present invention also concerns methods of using these modified oligonucleotides and kits containing the same.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

DESCRIPTIONMethods for Detecting and Amplifying Nucleic Acid Sequences Using Modified Oligonucleotides Having Increased Target Specific T_m Field of the Invention

This invention pertains to methods and compositions for detecting and amplifying nucleic acid sequences using oligonucleotides which contain one or more nucleotides
5 having a modification or modifications resulting in increased target affinity. Such oligonucleotides have been unexpectedly discovered to hybridize to a target nucleic acid at a significantly greater rate than a corresponding unmodified oligonucleotide hybridizes to the
10 same target. As a result, the methods and compositions of the present invention offer advantages for applications employing nucleic acid hybridization, such as medical and veterinary diagnostics, food testing and forensics.

Background of the Invention

15 In recent years, nucleic acid hybridization has become an increasingly important means of identifying, measuring and detecting the presence of particular nucleic acids in a given sample. Thus, for example, the fields of medical diagnostics, environmental and food testing, and
20 forensics have all benefitted from the use of nucleic acid hybridization as a rapid, simple and extraordinarily accurate way of testing for the presence or absence of given biological contaminants or microorganisms in a sample.

Most nucleic acid hybridization schemes have features
25 in common. One such typical feature is the use of single-stranded nucleic acid probes (or denatured double-stranded probes) having a defined or known nucleotide sequence. Probe molecules may be derived from biological sources, such as genomic DNA or RNA, or may be enzymatic-
30 ally synthesized, either in a prokaryotic or eukaryotic

host cell or in vitro. Presently, most nucleic acid probes in common use are oligonucleotide probes made using chemical synthetic methods ("synthetic oligonucleotides"). One such synthetic method is automated sequential addition of 3'-activated, protected nucleotides to the 5' end of a growing, solid phase-bound oligonucleotide chain, followed by cleavage of the completed oligonucleotide from the support and deprotection. See, e.g., Eckstein, Oligonucleotides & Analogues: A Practical Approach (1991).

10 Synthetic oligonucleotides for use as hybridization probes are typically deoxyribonucleotides having a nucleotide sequence complementary to a nucleotide sequence of the nucleic acid to be detected. DNA oligonucleotides are classically preferred for a number of reasons. Among
15 these is the greater stability DNA has to enzymatic hydrolysis upon exposure to common samples, due to the almost ubiquitous presence in samples of various RNases. RNA is also known to be less chemically stable than DNA, e.g., RNA degradation is facilitated by the presence of base,
20 heavy metals. And compared to RNA, DNA is less prone to assume stable secondary structures under assay conditions. Such secondary structures can render oligonucleotides unavailable for inter-molecular hybridization. Nevertheless, RNA oligonucleotides may be used, even though they
25 are less preferred.

Nucleic acid hybridization exploits the ability of single-stranded nucleic acids to form stable hybrids with corresponding regions of nucleic acid strands having complementary nucleotide sequences. Such hybrids usually
30 consist of double-stranded duplexes, although triple-stranded structures are also known. Generally speaking, single-strands of DNA or RNA are formed from nucleotides containing the bases adenine (A), cytosine (C), thymidine (T), guanine (G), uracil (U), or inosine (I). The single-
35 stranded chains may hybridize to form a double-stranded structure held together by hydrogen bonds between pairs of complementary bases. Generally, A is hydrogen bonded to

T or U, while G or I is hydrogen bonded to C. Along the double-stranded chain, classical base pairs of AT or AU, TA or UA, GC, or CG are present. Additionally, some mismatched base pairs (e.g., AG, GU) may be present. Under appropriate hybridization conditions, DNA/DNA, RNA/DNA, or RNA/RNA hybrids can form.

By "complementary" is meant that the nucleotide sequences of corresponding regions of two single-stranded nucleic acids, or two different regions of the same single-stranded nucleic acid, have a nucleotide base composition that allows the single strands to hybridize together in a stable double-stranded hydrogen-bonded region under stringent hybridization conditions. When a contiguous sequence of nucleotides of one single stranded region is able to form a series of "canonical" hydrogen-bonded base pairs with an analogous sequence of nucleotides of the other single-stranded region, such that A is paired with U or T, and C is paired with G, the nucleotides sequences are "perfectly" complementary.

The extreme specificity of nucleic acid hybridization, which under some circumstances can allow the discrimination of nucleic acids differing by as little as one base, has allowed the development of hybridization-based assays of samples containing specific microorganisms, nucleic acids bearing given genetic markers, tissue, biological fluids and the like. Such assays are often able to identify nucleic acids belonging to particular species of microorganisms in a sample containing other, closely-related species. Nucleic acid hybridization assays can also specifically detect or identify certain individuals, or groups of individuals, within a species, such as in the forensic use of RFLP (restriction fragment length polymorphism) and PCR (polymerase chain reaction) testing of samples of human origin.

The use of oligonucleotides as a diagnostic tool in nucleic acid hybridization testing often involves, but need not involve, the use of a reporting group or "label"

which is joined to the oligonucleotide probe molecule, or both the probe and the target. Such a reporter group moiety may include, for example, a radioisotope, chemiluminescent or fluorescent agent, or enzyme joined to the oligonucleotide. The label is employed to render the probe capable of detection, particularly when the probe is hybridized to the target nucleic acid.

The majority of assay methods employing nucleic acids utilize a physical separation step in order to separate the probe:analyte hybrid from unbound probe. These assay methods are called "heterogeneous" assays. In nucleic acid hybridization assays, an analyte molecule is the target nucleic acid species sought to be detected, quantitated and/or identified. A "hybrid" is a partly or wholly double-stranded nucleic acid comprising two single-stranded nucleic acids, such as a probe and a target nucleic acid, having a region of complementarity resulting in intermolecular hydrogen bonding under assay and/or amplification conditions.

Assay methods utilizing a physical separation step include methods employing a solid-phase matrix, such as glass, minerals or polymeric materials, in the separation process. The separation may involve preferentially binding the probe:analyte complex to the solid phase matrix, while allowing the unassociated probe molecules to remain in a liquid phase. Such binding may be non-specific, as, for example, in the case of hydroxyapatite, or specific, for example, through sequence-specific interaction of the target nucleic acid with a "capture" probe which is directly or indirectly immobilized on the solid support. In any such case, the amount of probe remaining bound to the solid phase support after a washing step is proportional to the amount of analyte in the sample.

Alternatively, the assay may involve preferentially binding the unhybridized probe while leaving the hybrid to remain in the liquid phase. In this case the amount of probe in the liquid phase after a washing step is pro-

portional to the amount of analyte in the original sample. When the probe is a nucleic acid or oligonucleotide, the solid support can include, without limitation, an adsorbent such as hydroxyapatite, a polycationic moiety, a hydrophobic or "reverse phase" material, an ion-exchange matrix, such as DEAE, a gel filtration matrix, or a combination of one or more of these solid phase materials. The solid support may contain one or more oligonucleotides, or other specific binding moiety, to capture, directly or indirectly, probe, target, or both. In the case of media, such as gel filtration, polyacrylamide gel or agarose gel, the separation is not due to binding of the oligonucleotide but is caused by molecular sieving of differently sized or shaped molecules. In the latter two cases, separation may be driven electrophoretically by application of an electrical current through the gel causing the differential migration through the gel of nucleic acids of different sizes or shapes, such as double-stranded and single-stranded nucleic acids.

A heterogeneous assay method may also involve binding the probe to a solid-phase matrix prior to addition of a sample suspected of containing the analyte of interest. The sample can be contacted with the label under conditions which would cause the desired nucleic acid to be labeled, if present in the sample mixture. The solid phase matrix may be derivatized or activated so that a covalent bond is formed between the probe and the matrix. Alternatively, the probe may be bound to the matrix through strong non-covalent interactions, including, without limitation, the following interactions: ionic, hydrophobic, reverse-phase, immunobinding, chelating, and enzyme-substrate. After the matrix-bound probe is exposed to the labeled nucleic acid under conditions allowing the formation of a hybrid, the separation step is accomplished by washing the solid-phase matrix free of any unbound, labeled analyte. Conversely, the analyte can be bound to the solid phase matrix and contacted with labeled probe,

with the excess free probe washed from the matrix before detection of the label.

Yet another type of assay system is termed "homogeneous assay." Homogenous assays can generally take place in solution, without a solid phase separation step, and commonly exploit chemical differences between the free probe and the analyte:probe complex. An example of an assay system which can be used in a homogenous or heterogeneous format is the hybridization protection assay (HPATM) disclosed in Arnold, et al., U.S. Patent 5,283,174, in which a probe is linked to a chemiluminescent moiety, contacted with an analyte and then subjected to selective chemical degradation or a detectable change in stability under conditions which alter the chemiluminescent reagent bound or joined to unhybridized probe, without altering the chemiluminescent reagent bound or joined to an analyte:probe conjugate. Subsequent initiation of a chemiluminescent reaction causes the hybrid-associated label to emit light. This patent enjoys common ownership with the present application and is expressly incorporated by reference herein.

Competition assays, in which a labeled probe or analyte competes for binding with its unlabeled analog, are also commonly used in a heterogeneous format. Depending on how the system is designed, either the amount of bound, labeled probe or the amount of unbound, labeled probe can be correlated with the amount of analyte in a sample. However, such an assay can also be used in a homogeneous format without a physical separation step, or in a format incorporating elements of both a homogeneous and a heterogeneous assay.

The assay methods described herein are merely illustrative and should not be understood as exhausting the assay formats employing nucleic acids known to those of skill in the art.

Nucleic acid hybridization has been utilized in methods aimed at using oligonucleotides as therapeutic

agents to modify or inhibit gene expression within living organisms. In an example of such utilization, oligonucleotide "antisense" agents can be targeted specifically to an mRNA species encoding a deleterious gene product, such as a viral protein or an oncogene. See, e.g., Zamecnik and Stephenson, 75 Proc. Nat'l Acad. Sci. (USA), 280-284 (1978); Stephenson and Zamecnik, 75 Proc. Nat'l Acad. Sci. (USA), 285-288 (1978); and Tullis, U.S. Patent No. 5,023,243. Although Applicant does not wish to be bound by theory, it is thought that the RNA:DNA duplex which results from the binding of the antisense oligonucleotide to RNA targets may serve as a substrate for RNase H, an RNA-degrading enzyme present in most cells and specific for RNA contained in an RNA:DNA duplex. According to this model, the target RNA molecule is destroyed through hybridization to the antisense oligonucleotide. Variations of this general strategy exist, wherein, for example, the oligonucleotide has a structure conferring an enzymatic activity on the oligonucleotide, such as the RNase activity of so-called ribozymes. See, e.g., Goodchild, PCT Publication No. WO93/15194.

Because therapeutic antisense oligonucleotides are primarily designed to function in vivo, formulations for the delivery of such agents must not significantly inhibit normal cellular function. Thus, nuclease inhibitors, which can sometimes be included in in vitro diagnostic tests to prevent oligonucleotide degradation, are not suitable for use in vivo. This fact has resulted in the design of various oligonucleotides modified at the internucleotide linkage, at the base or sugar moieties, or at combinations of these sites to have greater nuclease resistance than unmodified DNA.

Thus, a number of oligonucleotide derivatives have been made having modifications at the nitrogenous base, including replacement of the amino group at the 6 position of adenosine by hydrogen to yield purine; substitution of the 6-keto oxygen of guanosine with hydrogen to yield 2-

amino purine, or with sulphur to yield 6-thioguanosine, and replacement of the 4-keto oxygen of thymidine with either sulphur or hydrogen to yield, respectively, 4-thiothymidine or 4-hydrothymidine. All these nucleotide analogues can be used as reactants for the synthesis of oligonucleotides. See, e.g., Oligonucleotides and Analogues: A Practical Approach, supra. Other substituted bases are known in the art. See, e.g., Cook, et al., PCT Publication No. WO92/02258, entitled Nuclease Resistant, Pyrimidine Modified Oligonucleotides that Detect and Modulate Gene Expression, which is incorporated by reference herein. Base-modified nucleotide derivatives can be commercially obtained for oligonucleotide synthesis.

Similarly, a number of nucleotide derivatives have been reported having modifications of the ribofuranosyl or deoxyribofuranosyl moiety. See, e.g., Cook et al., PCT Publication No. WO94/19023 entitled Cyclobutyl Antisense Oligonucleotides, Methods of Making and Use Thereof; McGee, et al., PCT Publication No. WO94/02501 entitled Novel 2'-O-Alkyl Nucleosides and Phosphoramidites Processes for the Preparation and Uses Thereof; and Cook, PCT Publication No. WO93/13121 entitled Gapped 2'-modified Oligonucleotides. These three publications are incorporated by reference herein.

Most oligonucleotides comprising such modified bases have been formulated with increased cellular uptake, nuclease resistance, and/or increased substrate binding in mind. In other words, such oligonucleotides are described as therapeutic gene-modulating agents.

Nucleic acids having modified nucleotide residues exist in nature. Thus, depending on the type or source, modified bases in RNA can include methylated or dimethylated bases, deaminated bases, carboxylated bases, thiolated bases and bases having various combinations of these modifications. Additionally, 2'-O-alkylated bases are known to be present in naturally occurring nucleic

acids. See, Adams, The Biochemistry of the Nucleic Acids, 7,8 (11th ed. 1993).

Summary of the Invention

This invention concerns diagnostic methods and compositions employing nucleic acid hybridization techniques. Applicant has surprisingly discovered that oligonucleotides, comprised of one or more modified nucleotides, which have increased binding affinity to a target nucleic acid having a complementary nucleotide sequence, will hybridize to the target nucleic acid at a faster rate than unmodified oligonucleotides. The inventions described herein are drawn to the use of oligonucleotides, wholly or partially so modified, in methods involving their use as, for example, hybridization assay probes, amplification primers, helper oligonucleotides, and oligonucleotides for the capture and immobilization of desired nucleic acids.

Although the present invention is not to be seen as so limited, in particularly preferred embodiments the present invention concerns diagnostic methods utilizing oligonucleotides having nucleotides with 2' modifications to their ribofuranosyl (or deoxyribofuranosyl) moieties. In particular, Applicant has discovered that incorporating nucleotides having such modifications as part of synthetic oligonucleotides can profoundly increase the rate of nucleic acid hybridization, and the preferential binding of such oligonucleotides, to RNA targets over DNA targets.

A currently preferred embodiment makes use of oligonucleotides containing nucleotide analogues having 2'-O-methylribofuranosyl moieties linked to a nitrogenous base. Other substitutions at the 2' position of the sugar would be expected, in light of the present disclosure, to have similar properties so long as the substitution is not so large as to cause steric inhibition of hybridization.

Additionally, in light of the discoveries giving rise to the present invention, other modifications which increase the T_m of a modified oligonucleotide:target hybrid

would reasonably be expected to contribute to increases in the rate of hybridization as well. Such modifications may occur at the 2' position (or other positions) of the deoxyribofuranosyl or ribofuranosyl moiety (such as 2' halide substitutions), on the nitrogenous bases (such as N-diisobutylaminomethylidene-5-(1-propynyl)-2'-deoxycytidine; a cytidine analog, or 5-(1-propynyl)-2'-deoxyuridine); a thymidine analog, or in the linkage moiety. Thus, while specific reference is made throughout this application to 2' modifications, those of skill in the art will understand that other modifications leading to an increased T_m of a modified oligonucleotide:target hybrid over a hybrid containing an unmodified oligonucleotide of identical base sequence would be expected to have similar properties and effects on hybridization kinetics.

While the term T_m refers to the temperature at which 50% of a population of equal amounts of complementary nucleic acid strands are in the double-stranded form, throughout this disclosure the " T_m of an oligonucleotide" or a nucleic acid (single-stranded) is intended to mean the T_m of an oligonucleotide or nucleic acid in a nucleic acid duplex with a target, wherein the nucleic acid contains a base sequence region which is exactly complementary to a base sequence region of the oligonucleotide, unless otherwise indicated.

Because the T_m of the modified oligonucleotides is higher than that of corresponding, unmodified oligonucleotides of the same base sequence, the compositions and diagnostic methods described herein enable the use of oligonucleotides and oligonucleotide probes of shorter length than are otherwise practical for the specific hybridization and detection of nucleic acid targets (preferably RNA targets). The use of shorter oligonucleotides to specifically bind to target nucleic acids at a given temperature has additional advantages. For instance, shorter oligonucleotides will generally have a greater ability to discriminate perfectly complementary

targets from "mismatched" base sequence regions. Shorter oligonucleotides are also less likely to overlap undesirable base sequences. Additionally, because of the higher T_m , the modified oligonucleotides can stably hybridize at
5 higher temperatures than their unmodified counterparts.

The use of higher hybridization temperatures kinetically drives the hybridization reaction, resulting in faster hybridization rates than would occur at lower temperatures. Further, the modified oligonucleotides used
10 in the methods of the present invention result in faster hybridization rates than the unmodified versions, even when the temperature is not raised.

An increased hybridization rate leads to several other advantages in diagnostic assays. For example,
15 diagnostic assays conducted in accordance with the present invention can be conducted more rapidly than in previously existing hybridization assays. In cases in which the results of the assay may dictate a course of medical treatment or other action, a faster assay result has clear
20 prognostic advantages and may result in more effective treatment. Also, owing to faster hybridization rates and greater affinity of modified oligonucleotides for targets, especially RNA targets, lower concentrations of probe may be used to achieve the same amount of signal. Thus, the
25 assay background (or "noise") can be reduced and the lower concentration of probe can help eliminate undesirable cross-reactions with non-target nucleic acids. Additionally, these assays may be run in larger volumes of sample, thus increasing the sensitivity of the assay.

30 Thus, hybridization assay probes, amplification oligonucleotides, sample preparation oligonucleotides and/or helper oligonucleotides can all be designed to contain modified bases which have the advantage of increasing the rate of target-specific hybridization.

Brief Description of the Drawings

Figure 1 provides the IUPAC nomenclature for a sampling of acridinium esters that may be used as detectable chemiluminescent labels in the present disclosure.

5 Figure 2 shows an arrangement whereby detection of an analyte first requires the hybridization of the analyte to a nucleic acid other than the probe. According to this arrangement, the probe is unable to bind with either the analyte or the non-probe nucleic acid before the analyte
10 has hybridized to the non-probe nucleic acid. (Bolded portions represent regions of complementary between the analyte and the non-probe nucleic acid.) However, hybridization of the analyte to the non-probe nucleic acid alters the configuration of the non-probe nucleic acid
15 sufficiently to enable hybridization of the non-probe nucleic acid to the probe, thus permitting detection of the analyte.

Figure 3 shows the melting curve of a 2'-O-methyl oligonucleotide probe with either an RNA target or a DNA
20 target (two independent experiments), where melting is shown as an increase in light absorbance at 260nm (hyperchromatic shift).

Figure 4 shows hybridization of a single concentration of acridinium ester-labeled deoxy- or 2'-O-methyl
25 oligonucleotides of identical base sequence to varying amounts of a fully complementary RNA target during a fixed time of hybridization.

Figure 5 shows hybridization of varying amounts of acridinium ester-labeled deoxy- or 2'-O-methyl oligo-
30 nucleotides of identical base sequence to fixed amounts of a fully complementary RNA target during a fixed time of hybridization.

Figure 6 shows hybridization of a fixed amount of acridinium ester-labeled deoxy- and 2'-O-methyl oligo-
35 nucleotides of identical base sequence to a fixed amount of a fully complementary RNA target for various times of hybridization.

Figure 7 shows hybridization of a DNA or 2'-O-methyl oligonucleotide probe to a fully complementary RNA target. The data are plotted according to the equation $\ln(1-H) = (k)(C_0t)$, where H is the percent hybridization, k is the hybridization rate constant, C_0 is the concentration of probe, and t is time.

Detailed Description of the Invention

Definitions

Unless clearly indicated otherwise, the following terms will have the indicated meanings throughout this specification.

By "nucleic acid analyte" or "analyte" is meant a nucleic acid sought to be detected in a sample or a nucleic acid synthesized as a result of a nucleic acid amplification reaction which contains at least about 20 nucleotides of the nucleotide base sequence of a nucleic acid sought to be detected in a sample or the complement thereof.

By "synthesizing" a nucleic acid or oligonucleotide is meant making the nucleic acid by chemical synthesis or enzymatic means. It is known that certain nucleic acid polymerase enzymes can incorporate modified nucleotides during enzymatic synthesis.

By "modified", a "modified nucleotide" or "modification" is meant a purposeful variant from the classical ribo- and deoxyribonucleotides A, T, G, C and U. When used in this specification, modified will mean a variant of the classical nucleotides, said variants leading to a higher binding efficiency when an oligonucleotide which contains said modified nucleotides is hybridized to a target nucleic acid than when the same oligonucleotide contains the classical nucleotides. In some cases an oligonucleotide having a modified 3' end may be referred to. This means that the 3' end of the oligonucleotide contains a substitution which inhibits or prevents extension of the 3' end by a nucleic acid polymerase.

By "conjugate molecule" is meant a molecule that can couple with an oligonucleotide in such a way that at least some of the characteristics of both the molecule and the oligonucleotide are retained in the combined product.

5 Most often, the conjugate molecule contributes a new physical or chemical property to the oligonucleotide, while the oligonucleotide retains its ability to base pair.

By "binding affinity" is meant a measure of the
10 strength of hydrogen bonding between at least partly complementary nucleic acids under defined nucleic acid hybridization conditions. A convenient measure of binding efficiency is the T_m , which is the temperature at which 50% of said two strands are in the double-stranded or
15 hybridized form.

By "label" is meant a reporter moiety which is capable of being detected as an indication of the presence of the oligonucleotide to which it is joined. When the labeled oligonucleotide is hybridized to one or more other
20 oligonucleotides, the presence of the label can be an indication of the presence of the other oligonucleotide or oligonucleotides as well. Appropriate reporter moieties are well known in the art and include, for example, radioisotopes, dyes, chemiluminescent, fluorescent, chemi-
25 luminescent and electrochemiluminescent compounds, nucleic acid sequences, enzymes, enzyme substrates, chromophores and haptens.

By "nucleic acid assay conditions" is meant environmental conditions, including temperature and salt concentration, for the preferential formation of stable hybrids
30 between complementary base sequence regions over the formation of stable hybrids between non-complementary base sequence regions.

By "acridinium ester derivative" or "AE" is meant any
35 of a family of chemiluminescent compounds derived from the acridinium ring which have a labelled ester or ester-like linkage at the C9 position connecting the acridinium ring

to a leaving group. The leaving group is preferably an aryl or substituted aryl group. Substitutions, such as alkyl (e.g., methyl), alkoxy (e.g., methoxy), aryl and halide (e.g., Br and F), may be made to either or both the acridinium ring or the leaving group. Examples of such acridinium esters are provided in Figure 1.

The methods and compositions of the present invention result from the unexpected discovery that oligonucleotides containing one or more nucleotides modified so that the oligonucleotides have an increased T_m for a given target (as compared to otherwise identical unmodified oligonucleotides) will hybridize to a given target at an increased rate as compared to unmodified oligonucleotides. A maximum increase in the hybridization rate of a modified oligonucleotide occurs when a "cluster" of nucleotides are modified. By "cluster" is meant that at least about 4 of 5 consecutive nucleotides are so modified. Thus, oligonucleotides containing a mixture of modified and unmodified nucleotides may be just as effective in increasing target hybridization rate as in oligonucleotides containing 100% modified nucleotides. Aspects of the invention feature "chimeric" oligonucleotides containing both modified and unmodified nucleotides.

When used in this context, a "target" nucleic acid is a nucleic acid sought to be hybridized with an oligonucleotide. Such a nucleic acid may be a naturally occurring nucleic acid, e.g., ribosomal RNA, it may be the product (i.e., an "amplicon") of nucleic acid amplification methods such as PCR or a transcription-based amplification method, as described more fully below, or it may be another synthetic oligonucleotide.

Thus, the present invention is directed to diagnostic methods and compositions involving modified oligonucleotides which display an increase in the rate of oligo: target hybridization over an unmodified oligonucleotide of the same base sequence.

In a preferred embodiment, the invention utilizes modifications to the 2' position of the deoxyribofuranosyl (or ribofuranosyl) ring. The 2'-modification involves the placement of a group other than hydrogen or hydroxyl at the 2' position of the ribofuranosyl ring. Regardless of the nature of the substitution, it must not sterically hinder the ability of an oligonucleotide containing one or more such nucleotide modifications to hybridize to a single-stranded oligonucleotide having a complementary nucleotide base sequence. The hybridization of complementary, double-stranded nucleic acids in which one strand contains such modifications is markedly increased as compared to situations in which neither strand is so modified.

When a modified oligonucleotide is referred to as having an "increased" or "greater" affinity or rate, it is meant that the rate of hybridization or affinity of the modified oligonucleotide is greater than the hybridization rate or binding affinity of an unmodified oligonucleotide of the same length and base sequence to the same target.

Particularly preferred oligonucleotides are substituted with a methoxy group at the 2' sugar position. These 2'-modified oligonucleotides display a preference for RNA over DNA targets having a sequence identical to the RNA target (but having T substituted for U), with respect to both T_m and hybridization kinetics. Other such oligonucleotides are known in the art.

Conjugate molecules attached to oligonucleotides modified as described herein may function to further increase the binding affinity and hybridization rate of these oligonucleotides to a target. Such conjugate molecules may include, by way of example, cationic amines, intercalating dyes, antibiotics, proteins, peptide fragments, and metal ion complexes. Common cationic amines include, for example, spermine and spermidine, *i.e.*, polyamines. Intercalating dyes known in the art include, for example, ethidium bromide, acridines and proflavine.

Antibiotics which can bind to nucleic acids include, for example, actinomycin and netropsin. Proteins capable of binding to nucleic acids include, for example, restriction enzymes, transcription factors, and DNA and RNA modifying enzymes. Peptide fragments capable of binding to nucleic acids may contain, for example, a SPKK (serine-proline-lysine (arginine)-lysine (arginine)) motif, a KH motif or a RGG (arginine-glycine-glycine) box motif. See, e.g., Suzuki, EMBOJ, 8:797-804 (1989); and Bund, et al., Science, 265:615-621 (1994). Metal ion complexes which bind nucleic acids include, for example, cobalt hexamine and 1,10-phenanthroline-copper. Oligonucleotides represent yet another kind of conjugate molecule when, for example, the resulting hybrid includes three or more nucleic acids. An example of such a hybrid would be a triplex comprised of a target nucleic acid, an oligonucleotide probe hybridized to the target, and an oligonucleotide conjugate molecule hybridized to the probe. Conjugate molecules may bind to oligonucleotides by a variety of means, including, but not limited to, intercalation, groove interaction, electrostatic binding, and hydrogen bonding. Those skilled in the art will appreciate other conjugate molecules that can be attached to the modified oligonucleotides of the present invention. See, e.g., Goodchild, Bioconjugate Chemistry, 1(3):165-187 (1990). Moreover, a conjugate molecule can be bound or joined to a nucleotide or nucleotides either before or after synthesis of the oligonucleotide containing the nucleotide or nucleotides.

Applicant has also unexpectedly discovered that the observed increase in hybridization rate of modified oligonucleotides to their targets does not always increase indefinitely with an increasing number of contiguous modified nucleotides, especially when the target possesses an open or unfolded structure, or when helper probes are present. Under such circumstances, placement of modified nucleotides in a substantially contiguously arrangement,

i.e., about 4 of 5 contiguous nucleotides, in the oligonucleotide, followed by hybridization to a complementary target, will result in an increased hybridization rate only up to a given number of modifications. The addition
5 of modified nucleotides to the cluster past this number will not, in general, substantially further increase the hybridization rate.

In a currently preferred modification, employing 2'-O-methyl-substituted nucleotides, optimal hybridization
10 rates are obtained in oligonucleotides having a cluster of about 8 contiguous modified residues. Given the discovery that modified oligonucleotides can increase the hybridization rate, it was unexpected that this effect does not always parallel the increase in T_m contributed by such
15 additions. That is, addition of modified oligonucleotides past the rate-optimal cluster size will continue to increase the T_m .

Although Applicant does not wish to be limited by theory, it is believed that such clusters function as
20 "nucleation centers" which are the first regions of the oligonucleotide or nucleic acid to hydrogen-bond, in a rate-limiting step, followed by rapid hydrogen bonding of the remaining bases. While the entire oligonucleotide or nucleic acid may be so modified, little advantage in
25 increased hybridization rate appears to be gained by substantially exceeding the optimal cluster size.

However, when the structure of the target is closed or folded in nature, and no helper probes are included, the hybridization rate between the oligonucleotide and the
30 target generally may be improved by adding modified nucleotides to the oligonucleotide in excess of about 4 contiguous nucleotides. In a preferred embodiment, substantially all of the nucleotides of an oligonucleotide complementary to the structurally closed target will be
35 modified.

The rate of in-solution hybridization of two complementary single-stranded nucleic acids depends on various

factors, such as the concentration of the nucleic acids, the temperature of hybridization, and the properties of the solvent solution, such as salt concentration. Various methodologies have been employed to increase hybridization rates, the majority of which involve either changing the solvent system, such as by forming emulsions of immiscible solvents; by employing nucleic acid precipitating agents (e.g., Kohne et al., U.S. Patent No. 5,132,207), or volume excusion agents, such as polyethylene glycol; or by increasing the concentration of a nucleic acid strand.

A problem associated with the latter approach in a diagnostic assay is that the target nucleic acid is usually present in quite small amounts. Thus, to increase the nucleic acid concentration necessitates using an excess of the oligonucleotide, resulting in increased cost and reagent waste and, if the oligonucleotide is labeled, risking unacceptably high backgrounds. The other methods, such as those requiring the use of multiple solvents and agents, such as polyethylene glycol, may present practical difficulties, such as excessive sample manipulation and time.

Therefore, one aspect of the present invention provides a means for increasing hybridization rates, as well as binding affinity, of oligonucleotides for RNA targets by using oligonucleotides containing nucleotides having a substitution at the 2' position of the ribofuranosyl ring ("2'-modified oligonucleotide"); preferably an alkoxy substitution, most preferably a methoxy substitution. These properties render useful methods employing such oligonucleotides in a diagnostic hybridization assay format by increasing the rate and extent of hybridization of such an oligonucleotide without requiring a concomitant increase in the concentrations of the hybridizing nucleic acids, a change in the properties or composition of the hybridization solution, the addition of "helper oligonucleotides", disclosed in Hogan & Milliman, U.S. Patent No. 5,030,557, or an increase in the hybridization

temperature. U.S. Patent No. 5,030,557 enjoys common ownership with the present invention and is incorporated by reference herein. Nonetheless, the methods of the present invention may be used as supplements to one or more of these other techniques in any procedure in which an increase in the rate of nucleic acid hybridization would be advantageous.

As mentioned above, an advantage of this aspect of the invention relates to the ability of 2'-O-methyl modified oligonucleotides to preferentially hybridize to RNA over DNA. This property allows the design of oligonucleotide probes targeted to RNA. Probes can be made which would not tend to bind to DNA under stringent hybridization conditions, even where the DNA sequence is identical to the RNA target sequence (except that T is substituted for U in the DNA sequence). Such properties can be used in a number of different formats in which the specific detection of RNA would be advantageous. For example, to indicate and measure changes in the rate of transcription of particular RNA species, such as, for example, specific mRNA species, to monitor the effectiveness of a given therapy, to specifically probe tRNA or rRNA in preference to the genes encoding these RNA species, and to specifically detect RNA viruses in nucleic acid preparations containing large amounts of chromosomal DNA, even DNA preparations containing DNA versions of the viral sequences.

An additional advantage of this and other aspects is an increased target:oligo T_m when using modified oligonucleotides, such as 2'-modified nucleotides as compared to the T_m of target:oligo hybrids in which the oligonucleotide is a deoxyoligonucleotide. By "target:oligo" is meant a hydrogen bonded, double-stranded nucleic acid complex comprising a single-stranded oligonucleotide. The stability of the target:probe complex increases with an increase in the number of 2'-modified nucleotide residues contained in the probe. By contrast, the increase in the hybridization rate appears to be optimal in nucleic acids

having a cluster of about 8 2'-modified nucleotide residues and does not increase significantly upon the addition of consecutive modified oligonucleotide residues above that number. Further, "chimeric" oligonucleotides having
5 at least one such cluster of modified nucleotides could be designed to have greater hybridization rates without necessarily significantly increasing the T_m of the oligonucleotide as a whole to its target.

An increased T_m may be exploited in any diagnostic
10 procedure in which the added stability of a nucleic acid duplex is desired. For example, higher hybridization temperatures can be used to accelerate the hybridization rate. A higher T_m also permits the use of substantially shorter oligonucleotides than were heretofore practical,
15 thus resulting in a savings in the costs associated with producing oligonucleotides for hybridization, as well as other advantages, as mentioned above.

In other aspects and embodiments of the present invention, chimeric oligonucleotides may contain a modified portion designed to bind to target nucleic acid and
20 may also contain a deoxynucleotide portion which is either directly or indirectly able to bind to a solid phase-bound oligonucleotide. By way of example, and not of limitation, such an oligonucleotide may be a target capture
25 oligonucleotide designed to bind a target nucleic acid (e.g., in solution) and link the bound target nucleic acid to a derivatized solid phase matrix, such as a bead, microsphere, polymeric substance, such as agarose or dextran or to a magnetized particle. Derivatives linked
30 to such a matrix may include antibodies, ligands, or wholly or partially single-stranded oligonucleotides having a specific nucleotide sequence, such as a homopolymeric tract, designed to bind the capture oligonucleotide or an intermediate oligonucleotide. The bound
35 target can then be further hybridized with a probe (either RNA, DNA or modified) and the unbound probe washed free of

the immobilized target:probe complex before detecting the presence of the target nucleic acid.

It may be advantageous in certain instances to raise the temperature for hybridizing a modified oligonucleotide to its target. As described above, increasing the hybridization temperature also increases the rate of hybridization, so long as the hybridization temperature is sufficiently below the T_m of the desired hybrid. The hybridization methods claimed herein, employing modified oligonucleotides having a higher T_m than their unmodified counterparts, can be conducted at higher temperatures than would otherwise be used. In such a case, the rate increase associated with the modification alone is further increased by the raised temperature.

What follows are examples of embodiments of the invention, which should not be understood as limiting the scope of the invention thereto. Those skilled in the art will easily comprehend additional embodiments based on the disclosure contained in this specification. Additional embodiments are also contained within the claims which conclude this specification.

Modified Nucleic Acid Hybridization Assay Probes

Nucleic acid hybridization assays utilize one or more nucleic acid probes targeted to, *i.e.*, having a nucleotide base sequence substantially complementary to, a nucleic acid sought to be detected. Often the probe will comprise an oligonucleotide (a single-stranded nucleic acid of between about 10 and about 100 nucleotides in length) which is synthetically made to have a given nucleotide sequence. By "substantially complementary" is meant that the oligo will bind to its target under appropriately selective conditions to form a hydrogen bonded duplex.

While a hybridization assay probe will generally be joined to a detectable label, the probe can be unlabeled and probe:target hybrids detected by, for example, UV absorbance, HPLC chromatography, gel electrophoresis and

subsequent staining of the nucleic acid hybrid or other methods well known in the art. In hybridization assays, the probe and target are contacted with each other under conditions permitting stable and specific hybridization.

- 5 The resulting hybrid is then separated from unlabeled hybrid and the label detected, or the label can be detected under conditions allowing the detection of the hybrid in preference to the unlabeled probe.

Methods for separating nucleic acids, such as gel
10 exclusion chromatography, reverse phase chromatography, and hydroxyapatite adsorption, are known in the art. Applicant prefers an assay format wherein labeled hybrid and labeled unhybridized probe can be chemically differentiated by virtue of the formation of a double helix. A
15 particularly preferred assay format is the hybridization protection assay (HPA), see U.S. Patent No. 5,283,174 to Arnold et al., in which labeled unhybridized probe can be selectively be made undetectable while hybridized probe is relatively unaffected. Thus, detection of label in this
20 format is an indication of the labeled hybrid.

In these aspects, the present invention involves the use of modified oligonucleotides as probes in a hybridization assay. In one embodiment, modified oligonucleotides having a higher target-specific T_m than unmodified oligo-
25 nucleotides of the same base sequence are used to increase the rate of hybridization of the assay, as compared to assays employing unmodified oligonucleotides of the same base sequence. Such assay methods are able to utilize higher hybridization temperatures than are practicable
30 using unmodified oligonucleotides. The higher hybridization temperature further increases the rate of hybridization and can also reduce the amount of cross-hybridization (hybridization of the probe with non-target sequences), thereby increasing the specificity of the assay.

35 The probes of this invention may comprise a cluster of about 4 or more substantially contiguous, modified nucleotide residues mixed with unmodified residues.

Alternatively, the probe may comprise 100% modified residues. In preferred embodiments, the modifications are 2' substitutions, such as alkyl, alkoxy and halide substitutions to the 2' carbon of the ribofuranosyl nucleotide moiety. In particularly preferred embodiments, the substitution is a methoxy group.

Particular probe modifications, including 2'-O-methyl substitutions, result in the oligonucleotide having an increased affinity and increased hybridization rate to RNA targets but little effect on DNA affinity or rate of formation of probe:DNA hybrids. Again, this preference to RNA has been found to be optimized when the oligonucleotide has at least one cluster of from about 4 to about 8 modified bases.

Because oligonucleotides modified as described herein have a dramatically increased rate of hybridization, such modified oligonucleotide probes may be used in many cases without the need for the addition of unlabeled "helper probes", which are disclosed in Hogan, supra, as a means for increasing hybridization rates of probe to target. Nevertheless, Applicant has found that in some cases the combined use of such modified oligonucleotides and helper probes may operate in concert to increase the hybridization rate even further. In either case, the use of such modified oligonucleotides in diagnostic methods leads to more rapid identification of biological analytes, which in turn leads to, for example, to more effective treatments for disease conditions caused by or indicated by such analytes.

As described in more detail below, Applicant has found that probes displaying a preferential affinity to RNA targets may be used to specifically detect RNA over DNA of the same sequence (except that U is substituted for T in the RNA sequence). Such methods have application in, for example, the specific detection of RNA viruses in cells containing DNA versions of the viral genome, or in

specific detection of levels of RNA transcription in cells.

Probes may also be devised which contain both target-complementary sequences as well as additional target, non-complementary sequences. These target, non-complementary sequences may have other functions. For example, the sequences may be complementary to another oligonucleotide or target nucleic acid, or they may have functional properties, such as promoter sequences and restriction sites. Thus, a probe may have more than one function, only one of which is to be detected as an indication of the presence of a target.

Additionally, probes may be designed to have at least one nucleic acid strand which has at least two separate target-complementary sequences that can hybridize to a target nucleic acid. An example of such probes is described by Hogan *et al.*, U.S. Patent Nos. 5,424,413 and 5,451,503, which enjoy common ownership with the present application and are expressly incorporated by reference herein. The probes disclosed by Hogan *et al.* further include at least two distinct arm regions that do not hybridize with the target, but possess complementary regions that are capable of hybridizing with one another. These arm regions can be designed to require the presence of target in order for their complementary sequences to hybridize under suitable hybridization conditions. Accordingly, target-complementary sequences of the probe must hybridize to the target before complementary arm regions can hybridize to each other. The resulting structure is termed a branched nucleic acid.

Other probes may be designed which are unable to specifically bind the target. To be useful in detecting target, these probes must be able to hybridize with another nucleic acid that can bind with the target, either directly or indirectly. In one such arrangement, a nucleic acid can be structured to contain at least a first and second, nonoverlapping nucleotide base sequence

regions, where the first nucleotide region is complementary to a nucleotide base sequence of the target and the second nucleotide region is complementary to a nucleotide base sequence of the probe. In this arrangement, the second nucleotide region of the nucleic acid would be unavailable for binding with the probe until the target has hybridized with the first nucleotide region of the nucleic acid. Binding of the nucleic acid and target would alter the configuration of the nucleic acid, thus permitting the second nucleotide region of the nucleic acid to bind with the probe. See Figure 2. Of course, this arrangement could be modified so that indirect binding between the nucleic acid and target, accomplished with one or more intervening or coupling nucleic acids, would render the second nucleotide region of the nucleic acid available for binding with the probe.

Modified Nucleic Acid Amplification Oligonucleotides

In still other aspects, the present invention includes methods for employing modified oligonucleotide primers, promoter-primers, and/or splice templates for nucleic acid amplification and compositions comprising such oligonucleotides, wherein the oligonucleotides contain at least one cluster of modified bases which cause an increased rate of hybridization.

Primer-employing amplification methods include the polymerase chain reaction method (PCR) and its variations, as described by Mullis, et al., (see U.S. Patent Nos. 4,683,195, 4,683,202, and 4,800,159, European Patent Application Nos. 86302298.4, 86302299.2, and 87300203.4, and 155 Methods in Enzymology, 335-350 (1987)). The PCR methodology is by now a matter of common knowledge to those skilled in the art.

PCR has been coupled to RNA transcription by incorporating a promoter sequence into one of the primers used in the PCR reaction and then, after amplification by the PCR method, using the double-stranded DNA as a template

for the transcription of single-stranded RNA. (See, e.g., Murakawa, et al., DNA, 7:287-295 (1988)).

Other amplification methods use multiple cycles of RNA-directed DNA synthesis and transcription to amplify DNA or RNA targets. See, e.g., Burg, et al., U.S. Patent No. 5,437,990; 89/1050; Gingeras, et al., WO 88/10315; Davey and Malek, EPO Publication No. 0 329 822; Malek, et al., WO91/02818, Kacian and Fultz, U.S. Patent No. 5,480,783; McDonough, et al., WO 94/03472; and Kacian, et al., WO 93/22461 (the latter three of these publications enjoy common ownership with the present application and are incorporated by reference herein). Urdea, et al., WO91/10746, describe a method that achieves signal amplification using a T7 promoter sequence.

Each of these methods makes use of one or more oligonucleotide primers or splice templates able to hybridize to or near a given nucleotide sequence of interest. After hybridization of the primer, the target-complementary nucleic acid strand is enzymatically synthesized, either by extension of the 3' end of the primer or by transcription, using a promoter-primer or a splice template. In some amplification methods, such as PCR, rounds of primer extension by a nucleic acid polymerizing enzyme is alternated with thermal denaturation of complementary nucleic acid strands. Other methods, such as those of Kacian & Fultz, supra, McDonough, et al., supra, and Kacian, et al., supra, are isothermal transcription-based amplification methods.

In each amplification method, however, side reactions caused by hybridization of the primer to non-target sequences can reduce the sensitivity of the target-specific reaction. These competing "mismatches" may be reduced by raising the temperature of the reaction. However, raising the temperature may also lower the amount of target-specific primer binding as well.

Thus, according to this aspect of the invention, primers having high target affinity, and comprising

modified nucleotides in the target binding region, may be used in nucleic acid amplification methods to more sensitively detect and amplify small amounts of a target nucleic acid sequence, by virtue of the increased temperature, and thus the increased rate of hybridization to target molecules, while reducing the degree of competing side-reactions (cross-reactivity) due to non-specific primer binding. Preferred oligonucleotides contain at least one cluster of modified bases, but less than all nucleotides are modified in preferred oligonucleotides.

In another preferred embodiment, modified oligonucleotide primers are used in a nucleic acid amplification reaction in which a target nucleic acid is RNA. See, e.g., Kacian and Fultz, supra. The target may be the initially present nucleic acid in the sample, or may be an intermediate in the nucleic acid amplification reaction. In this embodiment, the use of preferred 2'-modified primers, such as oligonucleotides containing 2'-O-methyl nucleotides, permits their use at a higher hybridization temperature due to the relatively higher T_m conferred to the hybrid, as compared to the deoxyoligonucleotide of the same sequence. Also, due to the preference of such 2'-modified oligonucleotides for RNA over DNA, competition for primer molecules by non-target DNA sequences in a test sample may also be reduced. Further, in applications wherein specific RNA sequences are sought to be detected amid a population of DNA molecules having the same (assuming U and T to be equivalent) nucleic acid sequence, the use of modified oligonucleotide primers having kinetic and equilibrium preferences for RNA permits the specific amplification of RNA over DNA in a sample.

Sample Processing

In accord with the present invention, modified oligonucleotides having increased target-specific hybridization kinetics and binding affinities as compared to their

unmodified analogues may be used in a variety of hybridization assay sample processing methodologies.

By sample processing is meant methods allowing or enhancing the discrimination of analyte and non-analyte nucleic acids. Such methods may involve, for example, the direct or indirect immobilization of nucleic acids or oligonucleotides from the liquid phase in a heterogeneous assay. Some such methods may involve two or more hybridization events resulting in such immobilization.

For example, Ranki, et al., U.S. Patent Nos. 4,486,539 and 4,563,419, discuss a one-step nucleic acid "sandwich" hybridization method involving the use of a solid-phase bound nucleic acid having a target complementary sequence and a labeled nucleic acid probe, complementary to a separate portion of the target nucleic acid. Stabinsky, U.S. Patent No. 4,751,177, discusses methods involving the use of a "mediator" polynucleotide which reportedly overcomes sensitivity problems in the Ranki method associated with leakage of the immobilized oligonucleotide from the solid support.

Other methods may employ an immobilized oligonucleotide, for example an oligonucleotide containing a homopolymeric tract, such as poly T, or a simple short repeating sequence, and two or more coupling oligonucleotides, one of which is able to hybridize with the immobilized oligonucleotide and a different one of which is able to specifically hybridize with target. Each of the coupling oligonucleotides is able to bind at least one other coupling oligonucleotide. If a coupling oligonucleotide does not contain a sequence complementary to the target or immobilized oligonucleotide, it will be able to hybridize with at least two other coupling oligonucleotides simultaneously. The solid support may be comprised of materials including nitrocellulose, a polymeric substance, such as polyacrylamide or dextran, metallic substances or controlled pore glass. The support may be in forms such as a sheet, membrane or a particle.

Additionally, the solid support may have a magnetic charge to facilitate recovering sample and/or washing away unbound nucleic acids or other sample components.

Joining of the immobilized oligonucleotide to the solid support may be accomplished by any method that will continue to bind the immobilized oligonucleotide throughout the assay steps. Additionally, it is important that when the solid support is to be used in an assay, it be essentially incapable, under assay conditions, of the non-specific binding or adsorption of non-target oligonucleotides or nucleic acids.

Common immobilization methods include binding the nucleic acid or oligonucleotide to nitrocellulose, derivatized cellulose or nylon and similar materials. The latter two of these materials form covalent interactions with the immobilized oligonucleotide, while the former binds the oligo through hydrophobic interactions. When using these materials it is important to use a "blocking" solution, such as those containing a protein, such as bovine serum albumin (BSA), or "carrier" nucleic acid, such as salmon sperm DNA, to occupy remaining available binding sites on the solid support before use in the assay.

Other immobilization methods may include the use of a linker arm, for example, N-hydroxysuccinamide (NHS) and its derivatives, to join the oligonucleotide to the solid support. Common solid supports in such methods are, without limitation, silica, polyacrylamide derivatives and metallic substances. In such a method, one end of the linker may contain a reactive group (such as an amide group) which forms a covalent bond with the solid support, while the other end of the linker contains another reactive group which can bond with the oligonucleotide to be immobilized. In a particularly preferred embodiment, the oligonucleotide will form a bond with the linker at its 3' end. The linker is preferably substantially a straight-chain hydrocarbon which positions the immobilized

oligonucleotide at some distance from the surface of the solid support. However, non-covalent linkages, such as chelation or antigen-antibody complexes, may be used to join the oligonucleotide to the solid support.

5 A desirable embodiment of the latter assay system contains two coupling oligonucleotides: (i) a first coupling oligonucleotide containing a nucleotide sequence substantially complementary to the immobilized oligo-
10 nucleotide, for example, with a poly A nucleotide sequence complementary to a poly T sequence on the immobilized oligo, and (ii) a second coupling oligonucleotide containing a nucleotide sequence substantially complementary to the target nucleic acid, a detectably labeled probe, or both. In a preferred embodiment, the second coupling
15 oligonucleotide contains a nucleotide sequence substantially complementary to the target nucleic acid. Moreover, each coupling oligonucleotide in the preferred embodiment contains another nucleotide sequence which enables the first and second coupling oligonucleotides to
20 hybridize to each other under assay conditions. However, one or more additional coupling oligonucleotides may be introduced into the system, such that the first and second coupling oligonucleotides are indirectly bound to each other by means of these additional, intervening coupling
25 oligonucleotides. The additional coupling oligonucleotides would be substantially unable to hybridize with the any of the target nucleic acid, the detectably labeled oligonucleotide probe, or the immobilized oligonucleotide under assay conditions.

30 Yet another assay system having practical advantages in ease and rapidity of use may comprise an immobilized oligonucleotide having a portion complementary to a capturing oligonucleotide. The capturing oligonucleotide (capture probe) will contain a base sequence permitting
35 hybridization to the target. The capturing oligonucleotide will also have a label attached within or near the target-binding nucleotide sequence region, such as a

substituted or unsubstituted acridinium ester, which may be used in a homogeneous or semi-homogenous assay system to specifically detect hybrid nucleic acids without detecting single-stranded nucleic acids, such as the capture probe itself. Such a system favored by Applicant is the HPA™, which is discussed and incorporated by reference above. In the HPA™ format, the label contained on any capture probe which has not hybridized to its target will be hydrolyzed with the addition of base, while target:capture probe hybrid would protect the label associated therewith from hydrolysis.

An advantage to this latter assay system is that only one target-specific hybridization event (labeled capture probe:target) need occur for target detection, rather than two such events (capture probe:target and labeled probe:target) in the other sample processing procedures described herein. Fewer oligonucleotides in the assay would tend to make the assay faster and simpler to optimize, since the overall rate at which labeled target is captured is limited by the slowest hybridizing probe. Additionally, while the portion of the target complementary to the capturing oligonucleotide in these other assay systems does not have to be as specific as the target's probe binding region, this base sequence must be rare enough to avoid significant saturation of the capture probe with non-target nucleic acids. Thus, this preference for two separate and specific target sequences may place constraints on finding an appropriate target to which such assays are to be directed. By contrast, only one such target sequence need be found in the latter assay, since the same nucleotide sequence functions simultaneously to immobilize and detect the target nucleic acid.

Regardless of the approach used, a necessary element of any assay is a method of detection of the desired target. A number of options known to those of skill in the art are possible. One such option is the direct use

of a labeled nucleic acid probe. Such a probe would have a nucleotide sequence region which is specifically hybridizable with and substantially complementary to the target nucleic acid of interest. Upon hybridization to the target and immobilization of the target:probe hybrid, unbound probe can be washed away or inactivated and the remaining label hybrid-associated detected and/or measured.

Another option combines the elements of detection and nucleic acid amplification. In such a system, the target nucleic acid is immobilized as described, for example, and without limitation, in the assay procedures described above. One or more amplification oligonucleotides (see, e.g., Kacian, et al., WO93/22461), such as a primer, promoter-primer, or splice template, able to hybridize with a specific region of the target nucleic acid may be contacted with the immobilized target nucleic acid under nucleic acid amplification conditions, e.g., in the presence of one or more nucleic acid polymerases and ribo- and/or deoxyribonucleotide triphosphates.

The resulting polynucleotide strand (amplicon) can be made directly available for specific hybridization and detection with a labeled hybridization assay probe or for further amplification by hybridizing the polynucleotide strand with one or more additional amplification oligonucleotides under nucleic acid amplification conditions. If the latter option is chosen, the amplification reaction can be continued until the desired level of amplification is achieved, then the resulting amplicons, which may comprise copies of at least a portion of the immobilized target nucleic acid, polynucleotides complementary to at least a portion of the immobilized nucleic acid, or both, can be detected using one or more labeled oligonucleotide probes. If the amplification reaction is to take place while the target is immobilized, it is important that the portion of the target molecule to be used as a template for the amplicons not contain the nucleotide sequence region necessary for immobilization of the target nucleic

acid. Although amplicons of either or both senses can be detected with the labeled probes, in a preferred embodiment only amplicons of the opposite sense to, i.e., complementary to, the immobilized target are detected.

5 A heterogeneous target capture method such as this is particularly advantageous since crude clinical samples can contain substances which inhibit or interfere with the amplification reaction. Thus, the ability to separate the target nucleic acid from such interfering substances can
10 permit or enhance the sensitivity of nucleic acid amplification.

This solid-phase associated amplification scheme can be used in myriad assay systems, including those described above. Applicant currently prefers an assay system
15 employing one or more coupling oligonucleotides, as described above, which are able to indirectly link the target nucleic acid to the solid support. It is also preferred that the complementary nucleotide sequence regions of the support-coupled oligonucleotide, and the
20 capturing oligonucleotide designed to hybridize to it, be at least partially homopolymeric or contain simple repeating nucleotide sequences, so as to promote rapid hybridization. Additionally, or alternatively, these regions of either or both the immobilized oligonucleotide and the
25 capturing oligonucleotide may be modified in a manner consistent with the disclosure of this specification to increase the rate of hybridization between these oligonucleotides. In such a system, the target capture oligonucleotide and the target nucleic acids are preferably
30 allowed to hybridize in solution before hybridizing to the immobilized oligonucleotide. In the currently preferred embodiment, the immobilized target is washed, the amplification oligonucleotide (or oligonucleotides) is contacted with the immobilized target under nucleic acid
35 amplification conditions, and following amplification the labeled amplicon-directed probe is added and detected.

Applicant prefers to use the transcription-based amplification method described in Kacian & Fultz, supra, previously incorporated by reference. In accord with this method, a promoter-primer having a 3' region complementary to a portion of the target and a 5' region and a primer having the same nucleotide sequence as a portion of the target are contacted with a target RNA molecule. The primer and promoter-primer define the boundaries of the target region to be amplified, including both the sense present on the target molecule and its complement, and thus the length and sequence of the amplicon. In this preferred embodiment, the amplification oligonucleotides and immobilized target RNA are contacted in the presence of effective amounts of Moloney murine leukemia virus-derived reverse transcriptase and T7 RNA polymerase, both ribonucleotide and deoxyribonucleotide triphosphates, and necessary salts and cofactors at 42°C. Under these conditions, nucleic acid amplification occurs, resulting predominantly in production of RNA amplicons of a sense opposite to that of the target nucleic acid. These amplicons are then detected, e.g., by using an acridinium ester-labeled hybridization assay probe of the same sense as the target-nucleic acid, in the hybridization protection assay disclosed in Arnold, supra, previously incorporated by reference.

In this preferred embodiment, Applicant prefers that the 3' terminus of the immobilized oligonucleotide, target capture oligonucleotide and coupling oligonucleotide(s) be "capped" or blocked to prevent or inhibit their use as templates for nucleic acid polymerase activity. Capping may involve addition of 3'-deoxyribonucleotides (such as cordycepin), 3', 2'-dideoxynucleotide residues, non-nucleotide linkers, such as disclosed in Arnold, et al., supra, alkane-diol modifications, or non-complementary nucleotide residues at the 3' terminus.

Although Applicant currently prefers to contact the primers with the target following target immobilization,

there may be hybridization kinetic advantages to combining the target nucleic acid and at least one primer complementary thereto at the same time that the target capture oligonucleotide is added. Applicant believes that it is
5 advantageous to conduct the target hybridization in solution prior to immobilization of the target, as hybridization can take place in solution more rapidly than when one nucleic acid is immobilized.

Likewise, while Applicant prefers to form and detect
10 amplicons of the opposite sense to the target, there is no reason why one could not form and detect amplicons of either or both senses. Additionally, when amplifying target nucleic acids contained in crude clinical samples, it appears important to conduct a wash step prior to the amplifying step to prevent enzyme inhibition and/or nucleic
15 acid degradation due to substances present in the sample.

It will be clear to the skilled person that this methodology is amenable, either as described or with obvious modifications, to various other amplification
20 schemes including the polymerase chain reaction.

Modified Oligonucleotides in Sample Processing

Modified oligonucleotides able to hybridize to complementary targets with increased kinetics may be used in sample processing methods which employ nucleic acid
25 hybridization, including the target capture methods described above. In light of the present disclosure it will be apparent that such partly or wholly modified oligonucleotides may be employed as hybridization assay probes or amplification oligonucleotides in these systems.
30 Additionally, wholly or partially modified oligonucleotides having increased target-directed hybridization kinetics may be used as immobilized oligonucleotides, target capture oligonucleotides, and/or one or more coupling oligonucleotides in heterogenous assays employing
35 nucleic acid hybridization. For example, such modifications may be used to reduce the overall assay time or to

allow the hybridization steps of the assay to occur at a single temperature. The advantages of reduced time and ease of assay operation in a clinical setting gained thereby would be clear to those skilled in the art.

5 Additionally, oligonucleotides may be modified to have hybridization kinetics and/or equilibrium preferences for a specific type of nucleic acid, such as RNA or DNA. As disclosed above, for example, 2'-O-methyl oligonucleotides preferentially hybridize with RNA over DNA. Thus,
10 target capture oligonucleotides containing 2'-O-methyl nucleotides may be used to specifically capture RNA target nucleic acids, such as mRNA or rRNA, under hybridization conditions not promoting hybridization of the oligonucleotide to the genomic versions thereof. Likewise, 2'-O-
15 methyl-modified amplification oligonucleotides and/or labeled probes can be designed, thereby targeting RNA over DNA for amplification and/or detection, as described above.

Modified Helper Oligonucleotides

20 Helper oligonucleotides are described in the Hogan, supra. Helper oligonucleotides are generally unlabeled and used in conjunction with labeled hybridization assay probes to increase the labeled probe's T_m and hybridization rate by "opening up" target nucleotide sequence regions
25 which may be involved in secondary structure, thus making these regions available for hybridization with the labeled probe.

In light of the present disclosure, those of skill in the art will easily recognize that using modified helper
30 oligonucleotides which will hybridize with the target nucleic acid at an increased rate over their unmodified counterparts can lead to even greater hybridization rates of the labeled probe to their target. Thus, methods and compositions for detecting oligonucleotides employing such
35 modified helper oligonucleotides are intended to be encompassed within the scope of this invention. Preferred

helper oligonucleotides have modifications which give them a greater avidity towards RNA than DNA. In a preferred embodiment, such modifications include a cluster of at least about 4 2'-O-methyl nucleotides. In a particularly preferred embodiment, such modifications would include a cluster of about 8 2'-O-methyl nucleotides.

Diagnostic Kits

The methods described herein also clearly suggest diagnostic kits specially formulated for use in such methods. These kits will contain one or more oligonucleotides to be used in a diagnostic nucleic acid hybridization assay. At least one of these oligonucleotides will contain a cluster of at least about 4 modified nucleotides designed to hybridize to a target nucleic acid region at an increased rate over an otherwise identical oligonucleotide.

Such diagnostic kits may include, without limitation, one or any combination of the probe, amplification, helper and sample processing oligonucleotides described herein.

In a preferred embodiment of the present invention, the kit contains at least one labeled oligonucleotide probe having a region containing one or more clusters of at least about 4 contiguous 2'-modified nucleotide residues. In a more preferred embodiment, the region contains one or more clusters of about 8 2'-modified nucleotides.

Applicant currently prefers using an acridinium ester derivative as a non-radioactive label and the addition of a methoxy group as a 2' modification. In a particularly preferred embodiment, at least one of the modified oligonucleotides will comprise one or more clusters of at least about 4 2'-O-methyl nucleotides. Even more preferred is at least one oligonucleotide containing one or more clusters of about 8 2'-O methyl nucleotides.

Kits containing one or more of the modified oligonucleotides disclosed herein could be sold for use in any diagnostic hybridization assay method, or related amplifi-

cation method, of the present invention. In such an assay, at least one of the modified oligonucleotides contained in the kit would function as a probe able to hybridize to a target nucleic acid. If the modified probe is contacted with a sample containing the target nucleic acid, the probe will exhibit improved hybridization properties over an unmodified probe having an identical base sequence. For instance, the hybridization binding affinity between the target and the probe will be greater than the hybridization binding affinity between the target and an unmodified form of the probe, when subjected to the same hybridization assay conditions. Additionally, the hybridization rate between the target and the probe will be greater than the hybridization rate between the target and an unmodified form of the probe, when subjected to the same hybridization assay conditions.

To further improve the hybridization properties of the probe, one or more conjugate molecules may bound to the probe, preferably in a region containing a cluster of at least about 4 modified nucleotides. It is also expected that the kit would be packaged with instructions for using one or more modified oligonucleotides in a diagnostic hybridization assay of the present invention.

Objects

It is therefore an object of the present invention to provide methods for increasing the both the avidity of binding and the hybridization rate between a diagnostic nucleic acid probe and its target nucleic acid by utilizing probe molecules having one or more modified nucleotides, preferably a cluster of about 4 or more, and more preferably about 8, modified nucleotides. In preferred embodiments, the modifications comprise 2' modifications to the ribofuranosyl ring. In most preferred embodiments the modifications comprise a 2'-O-methyl substitution.

It is also an object to provide methods for increasing the rate of hybridization of a single-stranded

oligonucleotide to a target nucleic acid through the incorporation of a plurality of modified nucleotides into the oligonucleotide. An increased rate of hybridization accomplished in this manner would occur over and above the increase in hybridization kinetics accomplished by raising the temperature, salt concentration and/or the concentration of the nucleic acid reactants.

It is another object of the invention to provide diagnostic methods for selectively targeting RNA over DNA through the use of oligonucleotides modified to have an increased target binding efficiency and to hybridize to RNA at an enhanced rate over DNA. In a preferred embodiment, such oligonucleotides comprise a 2'-O-methyl modification to the ribofuranosyl ring.

It is an additional object of the invention to provide sample processing methods which employ an immobilized oligonucleotide to directly or indirectly capture target nucleic acids. In preferred embodiments, such methods employ one or more oligonucleotides which can specifically hybridize to the target nucleic acid, permitting its detection and immobilization. In a preferred embodiment, a single labeled oligonucleotide is responsible for both capture and detection of the target. In a particularly preferred embodiment, a coupling or bridging nucleic acid is bound to both the immobilized oligonucleotide and the oligonucleotide responsible for capture and detection of the target. Additional coupling nucleic acids are possible. Some or all of the oligonucleotides used in sample processing methods may contain modifications which accelerate the rate of target specific hybridization.

It is yet another object of the invention to provide target specific oligonucleotides of between about 10 and about 100 bases, preferably between about 10 and about 15 bases, and more preferably between about 12 and about 15 bases, which preferably contain at least one cluster of at least about 4 nucleotides, more preferably about 8 nucleotides, modified to increase their target-specific binding

efficiency while simultaneously increasing their discrimination between target and non-target nucleotide sequences as compared to longer unmodified oligonucleotides designed to hybridize to the same site.

5 It is a further object of the present invention to provide kits including one or more oligonucleotides containing modified nucleotides which function to increase the rate of hybridization between the oligonucleotide and a target nucleic acid. Kits of the present invention
10 could include any combination of probe, amplification, helper and sample processing oligonucleotides. In a preferred embodiment, the modified oligonucleotides of these kits would contain at least one cluster of about 4 2'-O-methyl modifications to the ribofuranosyl ring. Kits
15 containing these modified oligonucleotides may be supplied for use in both diagnostic hybridization assays and amplification assays. Such kits may further include written instructions directing practitioners in the use of the modified oligonucleotides in either or both diagnostic
20 hybridization assays or amplification assays.

 The diagnostic methods of the present invention are, therefore, specially adapted to exploit the hybridization properties of modified oligonucleotides having increased binding affinity. These methods may be used for the
25 detection or quantification of any target nucleic acid. In a preferred embodiment, the target nucleic acid is RNA. The methods may employ "chimeric" oligonucleotides composed of regions of unmodified oligodeoxy- or oligoribonucleotides combined with regions of modified oligonucleo-
30 tides or may utilize wholly modified oligonucleotides. Preferably, the oligonucleotides are not wholly modified. The regions may be designed simply to promote rapid hybridization of probe to target, or may have other functions. For example, a chimeric oligonucleotide may be
35 designed to bind both to RNA and to DNA. In such a case, the RNA-binding portion of the oligonucleotide may contain a plurality of modified nucleotides to preferentially bind

the RNA target. Alternatively, the region of modified residues may be designed to be directed towards a target present in low abundance in order to increase the hybridization rate.

5 Given the present disclosure, it will be understood that certain embodiments of the methods and compositions, including the kits, of the present invention may employ oligonucleotides having more than one type of modification affecting the hybridization properties of the resulting
10 oligonucleotide, i.e., T_m and hybridization kinetics. Such multiple modifications may act in a cooperative fashion to further increase the hybridization rate or to increase the specificity of the resulting oligonucleotide for a given type of nucleic acid target, such as RNA. Furthermore,
15 chimeric oligonucleotides may have or consist of regions of differently modified oligonucleotides containing either 2'- modified nucleotides or nucleotides having other modifications or both.

 The objects and aspects of the invention specifically
20 described herein are not intended as an exhaustive listing of the objects or aspects of the methods and compositions of the present invention which would be apparent to those skilled in the art in light of the present disclosure. Nor should the preceding description or the Examples which
25 follow be construed as limiting the invention to the embodiments specifically disclosed therein.

Examples

 Unless otherwise indicated, in all the following examples oligodeoxyribonucleotides, oligoribonucleotides,
30 and modified oligonucleotides were synthesized by use of standard phosphoroamidite chemistry, various methods of which are well known in the art. See e.g., Carruthers, et al., 154 Methods in Enzymology, 287 (1987), which is hereby incorporated by reference as part of this disclo-
35 sure. Unless otherwise stated herein, modified nucleotides were 2' O-methyl-nucleotides, which were used in the

synthesis as their phosphoramidite analogs. Applicant prepared the oligonucleotides using an Expedite 8909 DNA Synthesizer (PerSeptive Biosystems, Framingham, MA).

Also, unless otherwise indicated, oligonucleotides
5 indicated as labeled contained an acridinium phenyl ester.

Acridinium phenyl ester compounds are derivatives of acridine possessing a quaternary nitrogen center and derivatized at the 9 position to yield a phenyl ester moiety. However, leaving groups other than phenyl moieties are well known in the art. Acridinium esters have
10 the property of reacting with hydrogen peroxide to form a transient dioxetane ring involving the C-9 carbon of the acridinium ring, followed by the formation of an excited acridone. The radiative relaxation of the excited acridone results in the production of light. The synthesis of
15 acridinium esters, as well as a general description of their use as chemiluminescent labeling reagents, is described in Weeks, et al., Acridinium Esters as High Specific Activity Labels in Immunoassays, Clin. Chem.,
20 29:1474-1478 (1984), which is incorporated by reference herein.

In these Examples, the acridinium esters were attached, using standard chemical techniques, to a non-nucleotide monomeric unit having a primary amine "linker
25 arm" joined to the acridinium ester moiety, which is inserted between contiguous sequences of nucleotides during the chemical synthesis of the oligonucleotides, or placed at a terminal position of the oligonucleotide. See, Arnold, et al., Non-Nucleotide Linking Reagents for
30 Nucleotide Probes, EPO Publication No. EPO 313219, which enjoys common ownership with the present invention, and is now incorporated by reference herein. However, it will be understood that the preference of 2'-modified oligonucleotides for RNA targets and the effect of the modified
35 oligonucleotides on the rate of hybridization to DNA targets are not determined by the presence or specific nature of a label. Thus, those of skill in the art will

recognize that oligonucleotides used in the methods of the present invention may be labeled with a variety of labels, or they may be unlabelled when, for example, they are used as amplification primers, helper oligonucleotides or in a capture assay.

Acridinium ester derivatives may be joined to the linker arm: hybridization probe conjugate using techniques well known in the art. Preferably, Applicant uses the methods described in Nelson, et al., Detection of Acridinium Esters by Chemiluminescence in Non-Isotopic Probe Techniques (Academic Press 1992), and Arnold, et al., Non-Nucleotide Linking Reagents for Nucleotide Probes, EPO Publication No. EPO 313219, previously incorporated by reference herein.

Further, unless expressly indicated otherwise, all target nucleic acids were RNA.

It will nevertheless be clear to those of skill in the art, in light of the present disclosure, that other labels may be used in the methods and compositions of the present invention without departing from the spirit of the invention disclosed herein.

Example 1: Effect of 2' Modifications on the T_m of Probe:Target Hybrids

Oligonucleotide probes of identical sequence containing varying amounts of 2'-O-methyl nucleotides were each individually hybridized to perfectly complementary synthetic RNA targets of the same length. The probe sequence had SEQ ID NO:1 and the target sequence had SEQ ID NO:2. The nucleotide base sequences of these oligonucleotides were as follows:

SEQ ID NO:1 5'-GCTCGTTGCG GGA CTT(AE) AACC CAACAT-3'

SEQ ID NO:2 5'-ATGTTGGGTT AAGTCCCGCA ACGAGC-3'

The probes, as illustrated below, were synthesized to contain no 2'-O-methyl nucleotides (Probe A), all 2'-O-methyl nucleotides (Probe B), or a combination of deoxy- and 2'-O-methyl nucleotides (Probes C, D and E). Probe C

contained four contiguous deoxyribonucleotides positioned directly adjacent to each side of the linker attachment site and 2'-O-methyl ribonucleotides at all other bases; Probe D contained four contiguous 2'-O-methyl nucleotides positioned directly adjacent to each side of the linker attachment site and deoxyribonucleotides at all other bases, and Probe E contained eight contiguous 2'-O-methyl nucleotides positioned directly adjacent to each side of the linker attachment site and deoxyribonucleotides at all other bases. The T_m of each hybrid was determined using both a chemiluminescent and an optical method.

Chemiluminescent Method

Using the chemiluminescent method, approximately 1 pmol of the RNA target and 0.1 pmol of each oligonucleotide probe, labeled as described above with "standard" acridinium ester (4-(2-succinimidylloxycarbonyl ethyl) phenyl-10-methylacridinium 9-carboxylate fluorosulfonate) were allowed to hybridize at 60°C for 60 minutes in 30 μ l of lithium succinate buffer (1.5 mM EDTA (ethylenediamine-tetraacetic acid), 1.5 mM EGTA (ethylene glycol-bis (β -aminoethyl ether) N,N,N',N'-tetraacetic acid), 310 mM lithium lauryl sulfate, 0.1 M lithium succinate (pH 5.2)). The resulting solution was then diluted to 500 μ l with lithium succinate buffer, and 50 μ l aliquots were incubated at various temperatures for 7 minutes. Each sample was then cooled on ice for an additional 7 minutes. The acridinium ester coupled to unhybridized probe molecules was hydrolyzed by adding 150 μ l of a solution containing 190 mM $\text{Na}_2\text{B}_4\text{O}_7$ (pH 7.6), 7% (v/v) TRITON® X-100 (polyoxyethylene p-t-octyl phenol) and 0.02% (w/v) gelatin, and the samples were heated at 60°C for 10 minutes. The remaining (hybrid associated) chemiluminescence of each sample was determined in a LEADER® 50 luminometer (MGM Instruments; Hamden, Ct.) by the automatic injection of a solution containing 0.1% v/v H_2O_2 in 0.001 M HNO_3 , followed 0.5 - 2 seconds later by an injection of 200 μ l of 1N

NaOH. The resulting light emission was integrated over a 2 second interval.

Optical Method

Using the optical method, an identical set of oligo-
 5 nucleotide probes were synthesized having a linker arm but
 were not labeled with an acridinium ester. Four micro-
 grams of each oligonucleotide probe were allowed to
 hybridize to 4 μ g of the complementary RNA target for 60
 minutes at 60°C in 30 μ l of a hybridization buffer con-
 10 taining 200 mM lithium hydroxide, 3 mM EDTA, 3 mM EGTA,
 17% w/v lithium lauryl sulfate, and 190 mM succinic acid
 (pH 5.2). Following hybridization, 600 μ l of the hybridi-
 zation buffer was added, the sample split into two, and
 the melting behavior of each sample portion examined on a
 15 Beckman DU640 spectrophotometer equipped with a Micro T_m
 analysis accessory. The temperature was varied 1°C per
 minute for temperatures that were more than 10°C either
 lower or higher than the T_m and 0.5°C per minute at
 intervals of 0.2°C for all other temperatures. Changes in
 20 hypochromaticity were monitored and recorded as a function
 of temperature. Results are shown in Table 1 below.

Table 1

<u>Probe</u>	<u>Number of modified nucleotides</u>	<u>T_m (chemi- luminescent)</u>	<u>T_m (optical)</u>	<u>ΔT_m</u>
A	0	68	72.4	0
B	26	90	91.2	22; 18.8
C	18	83	87.9	15; 15.5
D	8	72	76.4	4; 4
E	16	nd	84.2	11.6

nd = not done

30 As shown in Table 1, the T_m data generated using the
 chemiluminescent and optical methods agreed well with each

other. The somewhat lower T_m values observed with the chemiluminescent method can be attributed to the lower nucleic acid concentrations used in the chemiluminescent method versus the optical method. The data show that replacement of all of the deoxyribonucleotide residues of Probe A with 2'-O-methyl nucleotides (Probe B) resulted in probe:RNA target hybrids having a T_m increased by about 20.4°C. Probes C, D and E exhibited T_m increases of 15°C, 4°C, and 11.6°C, respectively. By calculating the effect on T_m for each substitution of a 2'-O-methyl nucleotide, these data reveal that the T_m of the 2'-O-methyl oligonucleotide:RNA target hybrid increases about 0.8°C for every such replacement. This effect is approximately linear over the number of substitutions tested.

Example 2: Effect of 2'-Modified Nucleotides on T_m of Probe:rRNA Hybrids

Three sets of oligonucleotide probes of different length and sequence were synthesized, and each set contained two oligonucleotides of identical base sequence. Probe F was 17 bases in length and included an acridinium ester label joined at a site located between a thymine base and an adenine base. Probe G was 18 bases in length and likewise included an acridinium ester label joined at a site located between a thymine base and an adenine base. Probe H was 20 bases in length and included an acridinium ester label joined at a site located between a thymine base and a guanine base.

Each set of probes contained one oligonucleotide consisting entirely of deoxyribonucleotides and another oligonucleotide containing only 2'-O-methyl nucleotides. Each probe was then hybridized to the corresponding ribosomal RNA, and the T_m of the resulting hybrids determined by the chemiluminescent method described above. The results are shown in Table 2 below.

Table 2

Probe	Length (bases)	T_m (deoxy)	T_m (2' - O-methyl)	ΔT_m	ΔT_m per modified nucleotide
F	17	63	81	18	1.05
G	18	66	78	12	0.66
H	20	62	75	13	0.65

The data confirm the results of Example 1, showing that replacement of a deoxyribonucleotide with a 2'-O-methyl nucleotide increases the T_m of the resulting probe:RNA target hybrid. Additionally, when calculated as the average of the three probes' increase in T_m per modified nucleotide, the contribution of each modified nucleotide was an increase of 0.8°C per modified nucleotide.

Example 3: Effect of 2'-Modified Nucleotides on T_m of Probe:DNA Hybrids

In this Example, the effect of 2'-modification on probe:DNA targets was tested. Probe I, which contained varying amounts of 2'-O-methyl nucleotides, was hybridized to an exactly complementary DNA target of the same length and the melting behavior of the resultant hybrids examined by the chemiluminescent method described above. Probe I was 29 bases in length and included an acridinium ester label joined at a site located between a thymine base and a guanine base.

Probe I was designed to consist of: (i) all deoxyribonucleotides; (ii) all 2'-O-methyl nucleotides; and (iii) all 2'-O-methyl nucleotides except for four deoxyribonucleotides, which were positioned immediately on each side of the label attachment site. Results of the T_m determination are shown in Table 3 below.

Table 3

<u>Probe</u>	<u>Number of 2'-O-methyl nucleotides</u>	<u>T_m</u>	<u>Δ T_m per modified nucleotide</u>
I	0	69	0
I	29	77	0.28
I	21	75	0.29

As the data shows, replacement of deoxyribonucleotides with 2'-O-methyl nucleotides in Probes J and K caused the T_m of the labeled probe:DNA target to increase approximately 0.3°C per 2'-O-methyl residue.

10 A similar test was done using three sets of different oligonucleotides. Each set contained two oligonucleotides, one of the oligonucleotides containing deoxyribonucleotides and the other containing 100% 2'-O-methyl nucleotides, having identical base sequences. Probe J was
15 16 bases in length and included an acridinium ester label joined at a site located between a thymine base and an adenine base. Probe K was 18 bases in length and likewise included an acridinium ester label joined at a site located between a thymine base and an adenine base.
20 Probe L was 29 bases in length and included an acridinium ester label joined at a site located between a thymine base and a guanine base.

In each case the synthetic DNA targets were completely complementary to the probes. The results are
25 shown in Table 4 below.

Table 4

Probe	Number of 2'-O-methyl nucleotides	T_m (optical method)	ΔT_m per modified nucleotide
J	0	75.3	0
J	26	74.5	-0.03
K	0	71.6	0
K	19	67.0	-0.24
L	0	74	0
L	29	78.2	0.14

The data contained in Tables 3 and 4 demonstrates that the T_m of DNA targets is increased to a significantly lesser degree than RNA targets when 2'-O-methyl substitutions are introduced into the probes.

Example 4: Analysis of the Stabilities of Different Types of Nucleic Acid Hybrids

To compare the relative stabilities of hybrids containing various combinations of DNA, RNA, and 2'-O-methyl nucleotide strands, acridinium ester-labeled oligonucleotide probes of SEQ ID NO:1 (see Example 1 above) were hybridized to synthetic targets having a perfectly complementary base sequence. The probes and target sequences contained 100% ribonucleotides (RNA), 100% deoxyribonucleotides (DNA) or 100% 2'-O-methyl nucleotides in the combinations indicated in Table 5. The melting characteristics of each tested hybrid, as determined either using the chemiluminescent or the optical method, is shown in Table 5 below. More than one data point in the table indicates an independent, duplicate experiment.

Table 5

Probe	Target	<u>T_m (chemi-luminescent)</u>	<u>T_m (optical)</u>
DNA	RNA	68, 67	73.3, 73.6, 73, 72.4
RNA	RNA	81	nd
5 2'-O-methyl nucleotides	RNA	87, 90	91.2
2'-O-methyl nucleotides	2'-O-methyl nucleotides	91	nd
DNA	DNA	nd	75.5, 75.7, 75.1, 75.4
10 2'-O-methyl nucleotides	DNA	nd	74.3, 74.8

nd = no data

Thus, this experiment indicates that the stability of labeled probe:target hybrids follows the order: 2'-O-methyl/2'-O-methyl \geq 2'-O-methyl/RNA > RNA/RNA > DNA/DNA > 2'-O-methyl/DNA > DNA/RNA

Example 5: Ability of Enhanced Stability of 2'-Modified Oligo:RNA Hybrids to Allow Specific RNA Targeting

As indicated in Example 4, 2'-O-methyl:RNA hybrids are considerably more stable than 2'-O-methyl:DNA hybrids. To illustrate that this difference in stability can be exploited in a diagnostic assay to specifically detect RNA molecules over DNA molecules having an identical sequence (but with uracil in the RNA replacing thymine in the DNA), the following experiment was done.

An acridinium ester-labeled oligonucleotide probe of SEQ ID NO: 1 (see Example 1 above) and composed of 100% 2'-O-methyl nucleotides was allowed to hybridize to a completely complementary synthetic RNA or DNA target. Other than the fact that the oligonucleotide was labeled, hybridization and measurement of T_m were otherwise as described in Example 1 under the heading Optical Method.

The results are shown in Table 5 above and are further illustrated in Figure 3.

As indicated in Figure 3, at 81.6°C the 2'-O-methyl oligonucleotide forms a detectable hybrid with the RNA target, but not with the DNA target. By contrast, Table 5 demonstrates that when a labeled DNA oligonucleotide of the same sequence is hybridized with the identical RNA or DNA targets, the resulting hybrids have substantially similar melting characteristics.

Thus, according to the aspect of the present invention demonstrated here, it is possible to specifically detect RNA targets in preference to DNA targets under easily determined hybridization conditions. Such methods may be used, as a non-exclusive example, to specifically detect various RNA species, such as mRNA, tRNA, or rRNA, without interference from the identical sequence existing in the genomic DNA of the organism being assayed. Such methods may be useful for applications including monitoring the rate of expression of a particular gene product. Other uses exploiting this ability of the 2'-modified oligonucleotides will be apparent to those of skill in the art.

Example 6: Effect of 2'-Modified Nucleotides on the Hybridization Kinetics of Oligonucleotides

The effect of 2'-modified nucleotides on hybridization kinetics was illustrated using four different methods. The probe molecules used in this example were labeled with standard acridinium ester as described previously.

a) In the first approach, 2 fmol of an acridinium ester-labeled probe having SEQ ID NO: 1 (see Example 1 above) was hybridized to varying amounts of a completely complementary RNA target for a constant period of time, followed by differential hydrolysis and detection of the label. The hybridization was performed essentially as described in Example 1, under the heading Chemiluminescent

Method, with the following differences. Varying amounts of RNA target were allowed to hybridize with the labeled probe at 60°C for 45 minutes. Figure 4 shows the results of this experiment, wherein the probe was either a DNA oligonucleotide (open boxes) or consisted wholly of 2'-O-methyl nucleotides (closed diamond); these results are also tabulated in Table 6 below. The degree of hybridization is expressed in Relative Light Units (rlu), which is a measure of the number of photons emitted by the acridinium ester label.

Table 6

<u>Amount of Target</u> <u>(fmol)</u>	<u>rlu (DNA probe)</u>	<u>rlu (2'-O-</u> <u>methyl)</u>
1	855	1,739
5	3,394	8,009
10	5,476	14,217
50	13,810	27,959
100	18,798	34,381
200	19,199	31,318

The results indicate that the hybridization rate, as a function of target concentration, is significantly increased when the probe contains 2'-O-methyl nucleotides rather than unmodified nucleotides. This holds true throughout the range of target concentrations studied. For comparison purposes, the initial slopes of these data are used to estimate the relative hybridization rates of deoxy- (slope = 1.0) and 2'-O-methyl (slope= 2.5) oligonucleotide probes.

b) In a second approach, a constant amount (2 fmol) of the same target used in a) above was hybridized to varying amounts of the perfectly complementary probe for a fixed amount of time. Figure 5 shows the results of this experiment, wherein the probe was either a DNA oligonucleotide (open boxes) or consisted wholly of 2'-O-methyl

nucleotides (closed boxes). The hybridization and detection steps were the same as described in a) above, except that the hybridization reaction was carried out for 30 minutes rather than 45 minutes. The data are tabulated in Table 7 below.

Table 7

<u>Amount of Probe</u> <u>(fmoles)</u>	<u>rlu (DNA probe)</u>	<u>rlu (2'-O-</u> <u>methyl)</u>
0.2	99	346
0.5	309	966
1	690	1,973
2	1,206	4,356
5	3,227	9,184
10	5,801	14,615
20	7,289	21,515
50	13,080	33,236
100	15,223	34,930

Again, the results in this example indicate that the hybridization rate, which is a function of probe concentration, is significantly increased when the probe contains 2'-O-methyl nucleotides rather than unmodified nucleotides. The slopes of these plots are similar to those of Figure 4, indicating that regardless of whether probe concentration or target concentration is varied, the difference in hybridization kinetics between 2'-O-methyl/DNA and DNA/DNA interactions remains the same. In this experiment, the initial slope of the reaction containing the DNA probe was 1.0 and of the reaction containing the 2'-O-methyl probe was 3.1.

c) As a third illustration of the ability of 2'-modified oligonucleotides to increase the rate of hybridization, fixed amounts of either modified or unmodified probe (1 fmol) and target (100 amol) were allowed to

hybridize for varying amounts of time. The hybridization and detection protocols were otherwise the same as in b). Figure 6 shows the results, wherein the probe was either a DNA oligonucleotide (open boxes) or consisted wholly of 2'-O-methyl nucleotides (closed diamonds). The data were as follows in Table 8 below:

Table 8

<u>Time (minutes)</u>	<u>rlu (DNA probe)</u>	<u>rlu (2'-O-methyl)</u>
0	118	613
1	124	627
2	200	732
6.5	294	978
10.5	500	1331

Again, the relative rates of hybridization can be determined from the initial slopes of the curves (deoxy = 1.0; 2'-O-methyl = 2.2). In this experiment, the initial slope of the reaction containing the DNA oligonucleotide was 1.0, and the initial slope of the reaction containing the 2'-O-methyl oligonucleotide probe was 2.2-fold.

d) The fourth method used to demonstrate the differences between the hybridization kinetics of 2'-modified and unmodified probes was a C_0t analysis. Acridinium ester-labeled probes of SEQ ID NO:1 (see Example 1 above) were used. Either a fixed amount of probe and varied amounts of target ("probe excess") or a fixed amount of target and varying amounts of probe ("target excess") were allowed to hybridize at 60°C for varying amounts of time. The fixed amount of either probe or target was 0.25 fmol and the variable amount of either probe or target included amounts in the range from 0.25 to 50 fmol. Hybridization was otherwise as indicated in Example 1, under the heading Chemiluminescent Method.

Differential hydrolysis of the unhybridized acridinium ester and detection of the hybridized probe was

accomplished by adding 150 μ l of 190 mM $\text{Na}_2\text{B}_4\text{O}_7$ (pH 7.6), 7% (v/v) TRITON[®] X-100 and 0.02% (w/v) gelatin to the sample, and heating the mixture to 60°C for 10 minutes. Chemiluminescence was read in a luminometer, as described
5 above. Percent maximal hybridization was defined as the ratio of the observed rlu value divided by the maximal rlu value observed when saturating amounts of probe or target were used in the hybridization reaction.

In the C_0t plot shown in Figure 7, the quantity $\ln(1-H)$ is plotted against the concentration of target times the hybridization time. The value H is defined as the percent hybridization of the probe at a particular target
10 concentration after a particular time.

In this plot, the relative rates of hybridization of DNA and 2'-O-methyl oligonucleotide probes is given by the
15 inverse of the relative ratios of C_0t at 50% hybridization ($\ln(1-0.5)$; deoxy = 1.0 and 2'-O-methyl = 2.2).

A summary of the relative hybridization rates of an acridinium ester-labeled probe consisting entirely of
20 deoxy- or 2'-O-methyl ribonucleotides determined by these four methods is summarized in Table 9. The data resulting from these experiments indicate that at 60°C an oligonucleotide consisting entirely of 2'-O-methyl nucleotides hybridizes 2.3-fold faster than the corresponding deoxy-
25 ribonucleotide probe.

Table 9

Method	Probe (fmol)	Target (fmol)	Hybridization Time	Relative Rate (2'-O-methyl/ deoxy-)
c	0.05	1	vary	2.3
c	10	0.5	vary	2.3
c	0.1	1	vary	2.0
c	1	0.2	vary	1.6
c	3	0.3	vary	2.9
c	10	1	vary	1.8
b	vary	1	30	2.6
c	vary	2	45	3.1
a	2	vary	45	2.5
d	0.25	vary	44	2.2

Example 7: Kinetic Analysis of Hybridization of 2'-Modified Oligonucleotides to Additional Targets

To extend these hybridization rate comparisons to other probe and target sequences, Probe H of Example 2 above was synthesized entirely of either deoxy- or 2'-O-methyl nucleotides and hybridized to rRNA in the presence of helper probes. A C_0t analysis was performed, as in Example 6(d). The results are shown in Table 10 below.

Table 10

Probe	$C_0t_{1/2}$	Relative Rates
deoxy	0.3×10^7	1.00
2'-O-methyl	8×10^7	3.75

In this Example, the probe consisting entirely of 2'-O-methyl nucleotides hybridized 3.75-fold faster than the deoxyribonucleotide probe of identical sequence. Thus, enhanced hybridization by acridinium ester-labeled probes containing 2'-O-methyl nucleotides does not appear to be limited to any particular probe or target sequence.

Example 8: Effect of Increased Temperature on Hybridization Rate of 2'-Modified Oligonucleotides

As mentioned above, nucleic acid hybridization kinetics are accelerated by an increase in temperature. However, the advantages of this acceleration can be offset by the destabilizing effects of increased temperature on duplex formation, especially in diagnostic assays and nucleic acid amplification procedures employing relatively short oligonucleotides (between about 10 and about 50 bases in length). As shown below, the increased duplex stability provided by oligonucleotides modified as presently described can minimize this destabilizing effect, allowing the hybridization rate to be further increased by conducting the hybridization at a higher temperature than would otherwise be possible. Thus, a cooperative effect on hybridization kinetics is provided by the modified oligonucleotides and the higher hybridization temperature.

An acridinium ester-labeled oligonucleotide probe of SEQ ID NO:1 (see Example 1 above) consisting entirely of 2'-O-methyl nucleotides was allowed to hybridize to a perfectly complementary RNA target of the same length as described above. The hybridization and C_{ot} protocol were as described in Example 6(d), except that hybridization temperatures were either 60°C or 70°C. As shown by the data in Table 11 below, raising the temperature of hybridization of the 2'-O-methyl oligonucleotide to its target from 60°C or 70°C caused the hybridization kinetics to be accelerated 1.5 fold.

Table 11

<u>Hybridization Temperature</u>	<u>$C_{ot}_{1/2}$</u>	<u>Relative Hybridization Rates</u>
60°C	1.7×10^{-5}	1
70°C	1.1×10^{-5}	1.5

Example 9: Effect of Increasing Salt Concentration on Hybridization Kinetics of 2'-Modified Oligonucleotides

Hybridization kinetics are also accelerated by increases in salt concentration. The following example illustrates the effect of various concentrations of salt, e.g., LiCl, on the hybridization kinetics of 2'-O-methyl nucleotides. An acridinium ester-labeled probe of SEQ ID NO:1 (see Example 1 above), consisting entirely of 2'-O-methyl nucleotides, was allowed to hybridize, and a C_0t analysis conducted, as described in Example 6(d) above, at 80°C to an exactly complementary RNA target of the same length. Hybridization was performed at two different concentrations of LiCl. As shown in Table 12 below, increasing the salt concentration from 0.5 to 1.0 M LiCl enhanced the hybridization kinetics 2.9 fold.

Table 12

<u>LiCl Concentration</u>	<u>$C_0t_{1/2}$</u>	<u>Relative Rates</u>
0.5 M	0.72×10^{-5}	1
1.0 M	0.25×10^{-5}	2.9

These results demonstrate that a two-fold increase in the salt concentration in a hybridization reaction leads to a 2.9-fold increase in the hybridization kinetics of modified oligonucleotides.

Example 10: Combined Effect on Hybridization Kinetics of Increasing Salt Concentration and Temperature

To demonstrate the effect of simultaneously increasing the hybridization temperature and salt concentration on the hybridization kinetics of 2'-modified oligonucleotides, the following reactions were performed. An acridinium ester-labeled DNA oligonucleotide and an acridinium ester-labeled oligonucleotide having 100% 2'-O-methyl nucleotides, both of SEQ ID NO:1 (see Example 1 above), were each separately allowed to hybridize to an exactly complementary RNA target molecule in a C_0t analy-

sis. Hybridization conditions were as described in Example 6(d). Hybridization temperature and salt concentrations were as indicated in Table 13 below. The results were as follows:

5 Table 13

<u>Probe</u>	<u>Hybridiza- tion Temperature</u>	<u>LiCl Concentra- tion</u>	<u>C₀t_{0.5}</u>	<u>Relative Rates</u>
DNA	60°C	0.5 M	1.9×10^{-5}	1
2'-O-methyl	80°C	1.0 M	0.22×10^{-5}	8.6

10 As the data indicate, at a hybridization temperature of 80°C and in 1.0M LiCl, the 2'-O-methyl oligonucleotide hybridized to its target at an 8.6-fold faster rate than did the corresponding DNA oligonucleotide at a temperature of 60°C and salt concentration of 0.5M LiCl.

15 Example 11: Comparison of Hybridization Rates of RNA, DNA and 2'-Modified Oligonucleotides Hybridizing to Complementary DNA and RNA Targets

The relative hybridization rates of labeled RNA, DNA and 2'-O-methyl-containing oligonucleotides to completely
20 complementary DNA and RNA targets were individually determined. Rate determination was performed as disclosed in either Example 6(c) or 6(d) above. The labeled oligonucleotides had identical base sequences of SEQ ID NO:1 (see Example 1 above). The results are summarized in Table
25 14 below:

Table 14

<u>Probe</u>	<u>Target</u>	<u>Initial Slope</u>	<u>C₀t_{1/2}</u>	<u>T_m</u>	<u>Relative Rates</u>
DNA	DNA	.0031, .0048	---	75.4	4.4
RNA	DNA	---	---	74.5	---
2'-O Me	DNA	.0014, .0014	---	74.6	1.6
DNA	RNA	.0009	---	73.3	1
RNA	RNA	---	1.1 x 10 ⁻⁵ , 1.4 x 10 ⁻⁵	81	2.8
2'-O Me	RNA	.004	.71 x 10 ⁻⁵ , .89 x 10 ⁻⁵	91.2	4.4

Experiments represented in Rows 1 through 4 were done as disclosed in Example 6(c) using 3 fmol of labeled probe and 0.3 fmol of target. The experiments represented in rows 5 and 6 were performed using the method described in Example 6(d). Where more than one result is indicated, each value corresponds to a different individual experiment.

The results of Table 14 demonstrate that substitution of deoxyribonucleotide residues with either 2'-OH residues (RNA) or 2'-O-methyl residues enhances the affinity, as well as the hybridization kinetics, of the probe to an RNA target. In contrast, substitution of deoxyribonucleotide residues with 2'-O-methyl residues does not enhance the affinity or the hybridization kinetics of the probe to a DNA target.

The results presented in Table 14 reveal that substitution of deoxyribonucleotide residues with 2'-O-methyl residues enhances the affinity and hybridization kinetics of a probe for an RNA target, but not a DNA target. However, these experiments do not eliminate the possibility that the acridinium ester label and/or the linker, by which it is attached to the probe, may be responsible for these hybridization rate characteristics.

To show that the acridinium ester label and/or linker do not appreciably affect hybridization rates, the following experiment was performed.

An RNA probe of SEQ ID NO:1 labeled with acridinium ester (see Example 1 above) and containing a non-nucleotide linker by which the label was attached to the probe was allowed to hybridize to an exactly complementary target which consisted entirely of either 2'-O-methyl or deoxyribonucleotides. Hybridization and C_{ot} analysis was done as Example 6(d). The results are expressed in Table 15 below.

Table 15

<u>Labeled Probe</u>	<u>Target</u>	<u>$C_{ot}_{1/2}$</u>	<u>Relative Rates</u>
RNA	DNA	6.2×10^{-5}	1
RNA	2'-O-methyl	2.3×10^{-5}	2.7

These data reveal that substitution of deoxyribonucleotides with 2'-O-methyl residues enhances the hybridization kinetics of an oligonucleotide lacking an acridinium ester and/or linker. Thus, the increased hybridization rate observed for 2'-O-methyl modified probes is not due to the presence of a label or linker arm, but is an intrinsic property of the 2'-O-methyl-modified oligonucleotide.

Example 12: Comparative Effect of Helper Probes on the Hybridization of Probes from DNA Probe Mixes and 2'-Modified Probe Mixes to an rRNA Target

As disclosed in Hogan, *supra*, the hybridization of some probes to nucleic acids, especially those having a significant amount of secondary structure, is facilitated through the use of additional probes, termed "helper" probes. An example, though not the exclusive example, of a nucleic acid species having a high degree of secondary

structure is ribosomal RNA (rRNA). Helper probes can help disrupt secondary structure which may mask the target region. The helper probe is usually targeted to a sequence region near, but preferably not overlapping with, the probe target sequence. In practice, helper probes are generally not labeled, and are usually used in a large molar excess. The effect of using helper probes on labeled probe hybridization is usually expressed as an increased T_m and hybridization rate for the labeled probe:target hybrid.

The following experiments were performed to examine whether 2'-modified oligonucleotide probes have the same requirements for helper probes as do DNA probes when directed to given target regions containing a large amount of secondary structure. Two probe mixes were made. Each probe mix contained Probes F, G and H of Example 2 above. The oligonucleotides of one probe mix were made up of 100% 2'-O-methyl nucleotides, and the oligonucleotides of the other probe mix were composed entirely of deoxyribonucleotides. Where indicated, probe mixes included unlabeled DNA helper probes a, b, c, d, e and f having lengths of 33, 36, 41, 31, 29 and 40 bases, respectively.

Each helper probe was directed to rRNA base sequences close to the target site of one of the labeled probes. The degree of hybridization was measured using the hybridization protection assay (HPATM), as described above. The results are reported in relative light units (rlu).

As shown in Table 16 below, in the absence of helper probes, DNA probe mixes hybridized poorly to rRNA. In contrast, when probe mixes employing 2'-O-methyl probes of identical sequence to the DNA probe mixes were hybridized to rRNA in the absence of helper probes, much higher levels of hybridization were observed. Additionally, when helper probes were used with both probe mixes, significantly greater hybridization of the probes to their target occurred with the 2'-modified oligonucleotide probe mixes. Because the hybridization of a DNA probe to an rRNA

depends strongly on the presence of helper probes, while the hybridization of an identical 2'-O-methyl probe does not, 2'-O-methyl probes can efficiently hybridize to highly structured RNA molecules, such as ribosomal RNA, under conditions where DNA probes cannot.

Table 16

<u>Probe</u>	<u>Helpers</u>	<u>rRNA Concentration (amol)</u>	<u>rlus</u>
DNA	no	100	14
DNA	yes	100	3185
2'-O-methyl	no	100	3116
2'-O-methyl	yes	100	4332
DNA	no	1,000	68
DNA	yes	1,000	24,912
2'-O-methyl	no	1,000	19,934
2'-O-methyl	yes	1,000	33,584
DNA	no	10,000	730
DNA	yes	10,000	204,876
2'-O-methyl	no	10,000	148,386
2'-O-methyl	yes	10,000	256,940

The data in Table 16 further indicate that helper probes are not needed to facilitate probe binding when 2'-modified oligonucleotides are used. However, even greater sensitivity than seen before can be achieved in assays employing both helper probes and 2'-modified probes.

Example 13: Comparative Effect of Helper Probes on the Hybridization of a Single DNA Probe and a Single 2'-Modified Probe to an rRNA Target

The results of the experiments described in Example 12 were generated using three separate labeled probes, either with or without the presence of the six helper

oligonucleotides identified in Example 12. In this example, Probes F and H of Example 2, and consisting entirely of deoxyribo- or 2'-O-methyl nucleotides, were allowed to hybridize to target rRNA in the presence or absence of the indicated helper probes. Helper probes a, b, c and d of Example 12 above were used. Table 17 shows the hybridization characteristics of DNA and 2'-O-methyl oligonucleotides of Probe A. Table 18 shows the hybridization characteristics of DNA and 2'-O-methyl oligonucleotides of Probe H. Each labeled probe was tested in the presence or absence of the various helper probes and helper probe combinations indicated.

Table 17

<u>Probe</u>	<u>Target Concentration (amol)</u>	<u>Helper Probes</u>	<u>rlus</u>
deoxy	500	None	153
deoxy	500	a	5,600
deoxy	500	b	761
deoxy	500	a and b	9,363
2'-O-methyl	500	None	14,537
2'-O-methyl	500	a	16,556
2'-O-methyl	500	b	15,586
2'-O-methyl	500	a and b	16,868
deoxy	1,000	None	1,060
deoxy	1,000	a	14,782
deoxy	1,000	b	316
deoxy	1,000	a and b	26,877
2'-O-methyl	1,000	None	28,874
2'-O-methyl	1,000	a	23,201
2'-O-methyl	1,000	b	16,269
2'-O-methyl	1,000	a and b	44,510

Table 18

<u>Probe</u>	<u>Helper</u>	<u>RLUs</u>
deoxy	c	870
deoxy	d	9,176
deoxy	c and d	47,745
2'-O-methyl	c	88,292
2'-O-methyl	d	69,943
2'-O-methyl	c and d	98,663

Tables 17 and 18 demonstrate that the labeled DNA probes required helper oligonucleotides in order to effectively hybridize to their targets under the assay conditions. Additionally, Table 18 shows that the 2'-modified oligonucleotides hybridized to their targets to a greater degree in the absence of helper probes than the DNA oligonucleotides hybridized to the same target in the presence of the added helpers. Finally, 2'-O-methyl oligonucleotides exhibited even greater hybridization properties in the presence of helper oligonucleotides.

Example 14: Comparative Effect of Temperature on the Hybridization Properties of DNA Probes and 2'-Modified Probes with an rRNA Target in the Presence and Absence of Helper Probes

The data presented in Example 13 indicates that 2'-O-methyl oligonucleotides hybridize to a detectable extent to RNA targets, even highly folded structures like rRNA, in the absence of helper probes. Nevertheless, helper probes can accelerate the hybridization of 2'-O-methyl oligonucleotides to highly structured RNA. To examine the effect of helper probes more closely, deoxy- and 2'-O-methyl oligonucleotide probes were hybridized to rRNA at different temperatures in the presence or absence of helper probes. Table 19 represents studies performed using acridinium ester-labeled probes having a nucleotide

sequence of Probe F of Example 2 above and helper probes c and d of Example 12 above. Table 20 represents studies performed using acridinium ester-labeled probes having a nucleotide sequence of SEQ ID NO:1 (see Example 1 above) and helper probes g and h having 41 and 32 bases, respectively.

Table 19

<u>Probe</u>	<u>Helpers</u>	<u>Tempera- ture</u>	<u>C₀t_{1/2}</u>	<u>Relative Rate</u>
deoxy	Yes	60°C	26.4 X 10 ⁻⁵	1.3
2'-O-methyl	No	60°C	35 X 10 ⁻⁵	1
2'-O-methyl	Yes	60°C	8.15 X 10 ⁻⁵	4.3
2'-O-methyl	No	75°C	12.9 X 10 ⁻⁵	2.7
2'-O-methyl	Yes	75°C	4.12 X 10 ⁻⁵	8.5

Table 20

<u>Probe</u>	<u>Helpers</u>	<u>Tempera- ture</u>	<u>C₀t_{1/2}</u>	<u>Relative Rate</u>
2'-O-methyl	No	60°C	7.46 x 10 ⁻⁵	1
2'-O-methyl	Yes	60°C	1.77 x 10 ⁻⁵	4.2

These experiments demonstrate that at 60° and 75°C, helper probes enhanced the hybridization rates of the 2'-O-methyl probes to their targets 3.1-4.3 fold.

Example 15: Effect of 2'-Modified Nucleotides on the Hydrolysis Properties of Acridinium Ester-Labeled Probes

As a further demonstration of the effect of modified oligonucleotides on the performance characteristics of diagnostic probe molecules, a number of additional experiments were performed. These experiments were based on the Applicant's preferred detection method employing the HPA™ detection assay. In accordance with one HPA™ format, a

chemiluminescent acridinium ester is attached to a probe and the probe is hybridized to an analyte. Following hybridization, chemiluminescence associated with unhybridized probe is selectively destroyed by brief hydrolysis in borate buffer. Since probe:analyte molecules are not destroyed in this process, the remaining chemiluminescence of hybridized probe is a direct measure of the analyte present. In this application, those acridinium ester-labeled probes which hydrolyze faster when unhybridized than when in a probe-analyte hybrid complex are preferred. Hydrolysis of probe and hybrid is pseudo first order and can be characterized by the value $t_{1/2}$, which is the time, measured in minutes, required to hydrolyze 50% of the acridinium ester attached to either probe or hybrid. Thus, probes which exhibit a large differential hydrolysis (DH) ratio ($t_{1/2}$ (hybrid)/ $t_{1/2}$ (probe)) are highly desirable.

To examine the effect of modified oligonucleotides on the hydrolysis properties of acridinium ester-labeled probes, four sets of probes were constructed, each set having a distinct nucleotide base sequence and each member of a set having identical nucleotide base sequences. Each set of probes contained one probe consisting entirely of unmodified nucleotides and another probe consisting entirely of 2'-O-methyl nucleotides. The probes used were Probes A and B of Example 1 above and Probes F, G and H of Example 2 above. DH ratios of each probe to an exactly complementary RNA target were determined as described above, for example, in Example 1. As summarized in Table 21 below, unhybridized probes containing deoxy- or 2'-O-methyl nucleotides hydrolyzed at very similar rates.

Table 21

	Probe	Ribonucleotides	$t\%$ (Probe)	$t\%$ (Hybrid)	DH
A	A	deoxy	.81	49.1	60.3
	B	2'-O-methyl	.63	77.2	123.5
B	F	deoxy	.36	20.79	7.74
	F	2'-O-methyl	.89	75.25	4.55
C	H	deoxy	.69	17.26	25
	H	2'-O-methyl	.76	44.7	58.8
D	G	deoxy	.62	25.67	41.4
	G	2'-O-methyl	.81	23.55	29.7

In contrast, the modified probe:target hybrid-associated label for three of the sequences was approximately 2-fold more resistant to hydrolysis than in the otherwise identical unmodified probe:target hybrid. In one case, in which the probe contained an ATAT sequence surrounding the acridinium ester linker, the DH ratio was decreased 1.4-fold for the modified probe:target hybrid-associated label.

Example 16: Effect of Position of 2'-Modified Nucleotides on the Hydrolysis Properties of Acridinium Ester-Labeled Probe

To examine whether modified nucleotides must be close to the site of label attachment to enhance the DH behavior of acridinium ester, the acridinium ester-labeled probes of SEQ ID NO:1, containing clusters of 2'-O-methyl nucleotides at different positions relative to the acridinium ester linker site, were hybridized to a complementary RNA target. Those nucleotides containing 2'-O-methyl substitutions are indicated below by underlining:

Probe M: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'
 Probe N: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'
 Probe O: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'
 Probe P: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'

As shown in Table 22 below, the measurements reveal that 4 contiguous 2'-O-methyl nucleotides on either side of the acridinium ester linker site are sufficient to enhance the DH behavior of an acridinium ester-labeled probe as much as a probe consisting entirely of 2'-O-methyl nucleotides. More than one data point in the table indicates independent, duplicate experiments.

Table 22

Probe	t _{1/2} (Probe)	t _{1/2} (Hybrid)	DH
M	.82, .8	48.7, 43.2	59.7, 54
N	.76, .6	90, 77.6	118.3, 129
O	.74	49.8	67.3
P	.44	81.4	185

Example 17: Effect of Temperature on the Hydrolysis Properties of 2'-Modified Acridinium Ester-Labeled Probes

As mentioned above, because of their higher thermal stability, the modified oligonucleotides of the present invention are able to hybridize to a target nucleic acid at a higher temperature than unmodified oligonucleotides. At such higher temperatures, the hybridization rate, as well as the rate of other reactions, would be expected to increase. Among such other reactions is the rate of hydrolysis of acridinium ester labels. Because Applicant's preferred detection method employs the Hybridization Protection Assay (HPA™), described and incorporated by reference above, the following experiment was performed to determine whether benefits to the diagnostic assay conferred by an increase in hybridization rate would be offset by a decrease in the DH ratios of hybrid-associated and unassociated acridinium ester labels at this higher temperature.

An acridinium ester-labeled probe consisting entirely of 2'-O-methyl nucleotides was allowed to hybridize to a

complementary RNA target at 60°, 70° and 80°C. Hybridization conditions were as otherwise as described previously.

As summarized in Table 23 below, at 70°C and 80°C acridinium ester-labeled probes containing 2'-O-methyl nucleotides exhibited DH ratios comparable to acridinium ester-labeled probes containing deoxyribonucleotides at 60°C. Thus, elevated temperature may be used in diagnostic assays employing the methods and compositions of the present invention without a detectable decrease in assay sensitivity due to degradation of the label.

Table 23

Temp (°C)	Probe	$t\frac{1}{2}$ (Probe)	$t\frac{1}{2}$ (Hybrid)	DH
60	deoxy	.82	48.7	59.7
60	2'-O-methyl	.76	90	118
70	2'-O-methyl	.42	25.7	61.3
80	2'-O-methyl	.25	10.1	40.8

Example 18: Effect of 2'-Modified Nucleotides on the Hydrolysis Properties of Various Acridinium Ester-Labeled Probes

The foregoing experiments were conducted using standard acridinium ester as the detectable chemiluminescent label. To examine whether the differential hydrolysis behavior of labels other than standard acridinium ester is enhanced by T_m -enhancing modified nucleotides, probes of SEQ ID NO: 1 and containing either deoxy- or 2'-O-methyl nucleotides were labeled in exactly the same manner and position (see Example 1 above) with standard acridinium ester, o-diBr acridinium ester, 2-Me acridinium ester, naphthyl-acridinium ester, o-F acridinium ester, 2,7-diisopropylacridinium ester, or mixture of 1- and 3-Me acridinium ester, and their DH behavior examined.

See Figure 1 for examples of acridinium esters. As summarized in Table 24 below, the use of modified nucleotide

probes resulted in an increase in the DH ratio for all the acridinium ester derivatives tested by 1.1-6 fold.

Table 24

	<u>Label</u>	<u>Probe</u>	<u>t_{1/2}</u> <u>(Probe)</u>	<u>t_{1/2}</u> <u>(Hybrid)</u>	<u>DH</u>
5	Standard Acridinium ester	deoxy	.81	49.1	60.3
	Standard Acridinium ester	2'-O-methyl	.63	77.2	123.5
10	o-diBr-acridinium ester	deoxy	.94	23.44	24.94
	o-diBr-acridinium ester	2'-O-methyl	1.01	66.65	66
	2-Me-acridinium ester	deoxy	.72	73.6	87.62
15	2-Me-acridinium ester	2'-O-methyl	.78	101.8	130.5
	Naphthyl acridinium ester	deoxy	.72	14.45	20.07
20	Naphthyl acridinium ester	2'-O-methyl	.97	53.4	93.68
	o-F acridinium ester	deoxy	.93	53.3	57.3
	o-F acridinium ester	2'-O-methyl	1.03	78.75	76.46
	2,7-diisopropyl acridinium ester	deoxy	1.23	38.55	31.3
25	2,7-diisopropyl acridinium ester	2'-O-methyl	0.8	43.90	54.9
	Mixture of 1- and 3-Me acridinium ester	deoxy	.97	12.6	129.8
30	Mixture of 1- and 3-Me acridinium ester	2'-O-methyl	1.24	149.5	120.6

Example 19: Relationship Between the Hybridization Kinetics and the Number of 2'-Modified Nucleotides Contained in a Probe Sequence

To examine the relationship between hybridization kinetics and the number of 2'-O-methyl nucleotides within a probe sequence, acridinium ester-labeled probes of SEQ ID NO:1 (see Example 1 above) were synthesized. These synthesized probes contained increasing amounts of 2'-O-methyl residues (underlined bases indicate the presence of 2'-O-methyl residues) on both sides of the AE linker:

Probe Q: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'

Probe R: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'

Probe S: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'

Probe T: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'

Probe U: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'

Probe V: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'

The results are summarized in Table 25 below.

Table 25

<u>2'-O-methyl Residues</u>	<u>C₀t (Exp 1)</u>	<u>C₀t (Exp 2)</u>	<u>C₀t (Exp 3)</u>	<u>Relative Rate</u>
0	1.87 x 10 ⁻⁵	2.2 x 10 ⁻⁵	---	1
2	---	---	1.13 x 10 ⁻⁵	1.8
4	1.28 x 10 ⁻⁵	---	0.93 x 10 ⁻⁵	1.8
8	---	0.93 x 10 ⁻⁵	0.86 x 10 ⁻⁵	2.3
16	---	---	0.96 x 10 ⁻⁵	2.1
25	0.91 x 10 ⁻⁵	1 x 10 ⁻⁵	0.84 x 10 ⁻⁵	2.2

As summarized above, as few as 8 2'-O-methyl nucleotides (Probe T) -- 4 on each side of the acridinium ester linker site -- were sufficient to accelerate the hybridization rate of an acridinium ester probe to the same level of a probe consisting almost entirely of 2'-O-methyl nucleotides (Probe V). In contrast, the T_m of a probe containing four 2'-O-methyl nucleotides on each side of

the acridinium ester linker site is lower than the T_m of a probe:target hybrid in which the probe contains additional 2'-O-methyl nucleotides. Thus, according to the present invention it is possible to optimize, or "tune", the performance of an acridinium ester labeled probe with respect to its hybridization rate, differential hydrolysis, and melting properties. For example, an acridinium ester-labeled probe containing four 2'-O-methyl nucleotides on either side of the acridinium ester linker site will have its hybridization rate and differential hydrolysis properties maximally optimized, while a hybrid containing this probe will exhibit only a small increase in its melting temperature.

As substantially contiguous 2'-O-methyl nucleotides are added to replace deoxyribonucleotides in the labeled probe, the hybridization rate and differential hydrolysis properties of the probe:target hybrid will remain substantially constant while its T_m will continue to increase. The ability to increase, incrementally, the influence of a probe on the T_m of a probe:target hybrid allows one to adjust the specificity of a probe so as not to cross-react with closely related sequences, as shown in Table 25 above.

Example 20: Effect of Propyne-Modifications on the T_m of Probe:Target Hybrids

In order to illustrate the general usefulness of the compositions and methods of the present invention in the diagnostic application of nucleic acid hybridization technology, oligonucleotides were constructed having a modification other than a 2'-modification to the ribofuranosyl moiety, but which also caused an increase in the binding affinity of a probe for its target. In this example, oligonucleotides were synthesized containing two nucleotides modified at the nitrogenous base. Specifically, N-diisobutylaminomethylidene-5-(1-propynyl)-2'-deoxycytidine; (a cytidine analog) and 5-(1-propynyl)-2'-deoxy-

uridine) (a thymidine analog). These nucleotide analogs are commercially available, for example, from Glen Research in Sterling, VA.

As a first consideration, probes having 22 bases and an acridinium-ester attached at a site located between a thymine base and a guanine base in Probes W and Y and between two thymine bases in Probe X, but containing varying numbers of propyne-modified nucleotides, were hybridized to target rRNA in the presence of helper probes to examine the effect of the modification on the T_m of acridinium ester hybrids. Probe W contained no propyne modifications. Probe X contained two propyne modifications, one directly adjacent to each side of the label attachment site. Probe Y contained 11 propyne modifications, including four contiguous modifications directly adjacent and 5' to the label attachment site and seven modifications located at bases spaced 3, 4, 6, 9-11 and 14 bases away from and 3' to the label attachment site.

Hybridization and T_m determinations were performed as described above using detection of acridinium ester-labeled hybrids. As summarized below in Table 26, these data indicate that the T_m of the oligonucleotide, when hybridized to an RNA target, increased an average of 1°C for every replacement of a pyrimidine with a propyne-substituted pyrimidine.

Table 26

<u>Probe</u>	<u>Propyne Residues</u>	<u>T_m (chemi-luminescent)</u>	<u>ΔT/Propyne</u>
W	0	71	---
X	2	72	0.5
Y	11	82	1.0

Example 21: Effect of Propyne Modifications on the Hybridization Kinetics of Oligonucleotides

To examine the effect of propyne groups on the hybridization kinetics of oligonucleotides, the rate of hybridization of the propyne-labeled probes of Example 20 to RNA were examined by C_0t analysis, as described in Example 6. As summarized below in Table 27, the probe containing two propyne groups (Probe W) hybridized at the same rate as the probe containing no propyne groups (Probe X), while the probe containing 11 propyne groups (Probe Y) hybridized 1.9-fold faster.

Table 27

<u>Probe</u>	<u>C_0t_x</u>	<u>Relative Rate</u>
W	0.75×10^{-5}	1
X	0.81×10^{-5}	0.93
Y	0.39×10^{-5}	1.9

These data support the generality of the present invention by demonstrating that modifications to oligonucleotides which result in an increased T_m also cause the rate of hybridization of the modified oligonucleotide to its target to increase compared to an unmodified oligonucleotide of the same base sequence. Moreover, this example also demonstrates that such modifications may occur in the nitrogenous base moiety as well as the sugar moiety. Those of skill in the art will recognize that such modifications may also occur in the internucleotide linkage as well.

Although the foregoing disclosure describes the preferred embodiments of the present invention, Applicant should not be limited thereto. Those of skill in the art to which this invention applies will comprehend additional embodiments in light of this disclosure. Moreover, additional embodiments are within the claims which conclude this specification and their equivalents.

77

SEQUENCE LISTING

(1) GENERAL INFORMATION

- (i) APPLICANTS: Becker, Michael M.
Majlessi, Mehrdad
- 5 (ii) TITLE OF THE INVENTION: Methods for Detecting
and Amplifying Nucleic Acid Sequences Using
Modified Oligonucleotides Having Increased
Target Specific T_m
- (iii) NUMBER OF SEQUENCES: 2
- 10 (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: Gen-Probe Incorporated
- (B) STREET: 9880 Campus Point Drive
- (C) CITY: San Diego
- (D) STATE: California
- 15 (E) COUNTRY: USA
- (F) ZIP: 92121
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: 3.5" Disk, 1.44 Mb
- (B) COMPUTER: IBM Compatible
- 20 (C) OPERATING SYSTEM: MS-DOS Version 6.22
- (D) SOFTWARE: FastSEQ Version 1.5
- (vi) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Fisher, Carlos A
- (B) REGISTRATION NUMBER: 36,510
- 25 (C) REFERENCE/DOCKET NUMBER: GP95013
- (vii) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: 619-535-2807
- (B) TELEFAX: 619-546-7929

(2) INFORMATION FOR SEQ ID NO:1:

- 30 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- 35 (ii) SEQUENCE DESCRIPTION: SEQ ID NO:1:
- GCTCGTTGCG GGACTTAACC CAACAT

26

78

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGTTGGGTT AAGTCCCGCA ACGAGC

26

Claims

1. A method of increasing the hybridization rate between first and second nucleic acids in a diagnostic hybridization assay for use in detecting the presence or amount of at least one nucleic acid analyte, wherein a first nucleotide base sequence region of said first nucleic acid is able to stably hybridize to a first nucleotide base sequence region of said second nucleic acid under selective hybridization conditions, which comprises the steps of:

a) synthesizing at least one of said nucleotide regions to include one or more modified nucleotides, so that

i) the hybridization binding affinity between said first and second nucleic acids is greater than the hybridization binding affinity between unmodified forms of said first and second nucleic acids, under said conditions, and

ii) the hybridization rate between said first and second nucleic acids is greater than the hybridization rate between unmodified forms of said first and second nucleic acids, under said conditions; and

b) contacting said first and second nucleic acids of step a) under said conditions, such that said nucleotide regions are able to stably hybridize.

2. The method of claim 1, wherein said first and second nucleic acids are contained on the same nucleic acid strand.

3. The method of claim 1, wherein at least one of said nucleotide regions includes one or more clusters of at least about 4 modified nucleotides.

4. The method of claim 1, wherein at least one of said nucleotide regions includes one or more clusters of at least about 6 modified nucleotides.

5. The method of claim 1, wherein at least one of said nucleotide regions includes one or more clusters of at least about 8 modified nucleotides.

6. The method of claim 1, wherein substantially all
5 of the nucleotides contained in at least one of said nucleotide regions are modified.

7. The method of claim 3, wherein at least one of said modified nucleotides includes a modification selected from the group consisting of:

- 10 a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;
- c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage;
- and
- 15 e) a modification to the internucleotide linkage.

8. The method of claim 3, wherein at least one of said modified nucleotides includes two modifications selected from the group consisting of:

- 20 a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;
- c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage;
- e) a modification to the internucleotide linkage.

9. The method of claim 3, wherein at least one of
25 said modified nucleotides includes a 2'-modification to the ribofuranosyl moiety selected from the group consisting of:

- a) an alkyl substitution;
- b) an alkoxy substitution; and
- 30 c) a halide substitution.

10. The method of claim 3, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

11. The method of claim 3, wherein at least one of
5 said modified nucleotides includes a propyne substitution to the nitrogenous base.

12. The method of claim 11, wherein said propyne substitution is to a cytidine analog.

13. The method of claim 11, wherein said propyne
10 substitution is to a thymidine analog.

14. The method of claim 3, wherein each said modified nucleotide of one or more of said clusters of at least one of said nucleotide regions contains the same modification.

15. The method of claim 14, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

16. The method of claim 1, wherein at least one of said nucleic acids includes one or more conjugate
20 molecules.

17. The method of claim 3, wherein at least one of said nucleic acids includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said nucleic acids at
25 a site located within one or more of said clusters contained in at least one of said nucleotide regions.

18. The method of claim 3, wherein said first nucleotide region of said first nucleic acid includes one or more clusters of at least about 4 modified nucleotides.

19. The method of claim 18, wherein said first nucleic acid comprises an oligonucleotide probe.

20. The method of claim 19, wherein said probe consists of from about 10 to about 100 nucleotide bases.

5 21. The method of claim 19, wherein said probe consists of from about 10 to about 15 nucleotide bases.

22. The method of claim 19, wherein said probe consists of from about 12 to about 15 nucleotide bases.

10 23. The method of claim 19, wherein said probe further includes a label.

24. The method of claim 23, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b) an enzyme,
- 15 c) an enzyme cofactor,
- d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- 20 h) a fluorescent molecule,
- i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- l) a nucleotide base sequence region that is unable
- 25 to stably hybridize to said second nucleic acid under said conditions.

25. The method of claim 24, wherein said label is a chemiluminescent molecule.

26. The method of claim 25, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

27. The method of claim 25, wherein said chemiluminescent molecule is an acridinium ester derivative.

28. The method of claim 23, wherein said label is joined to said probe at a site located within one of said clusters contained in said first nucleotide region of said probe.

29. The method of claim 19, wherein said second nucleic acid comprises said analyte.

30. The method of claim 29, wherein said analyte consists of RNA.

31. The method of claim 30, wherein said RNA consists of rRNA or tRNA.

32. The method of claim 29, wherein said analyte consists of DNA.

33. The method of claim 3, wherein each said nucleotide region includes one or more clusters of at least about 4 modified nucleotides.

34. The method of claim 33, wherein each said modified nucleotide of one or more of said clusters of at least one of said nucleotide regions contains the same modification.

35. The method of claim 34, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

36. A method for detecting the presence or amount of an analyte comprising a first nucleic acid in a sample suspected of containing said analyte, which comprises the steps of:

- 5 a) contacting said sample with a probe comprising a second nucleic acid, wherein said probe contains a first nucleotide base sequence region that is able to stably hybridize to a first nucleotide base sequence region of said analyte under selective hybridization conditions, and
10 wherein said first nucleotide region of said probe includes one or more modified nucleotides;
- b) subjecting the components of step a) to said conditions, so that
 - 15 i) the hybridization binding affinity between said analyte and said probe is greater than the hybridization binding affinity between said analyte and an unmodified form of said probe, under said conditions, and
 - ii) the hybridization rate between said analyte and said probe is greater than the hybridization rate between
20 said analyte and an unmodified form of said probe, under said conditions; and
- c) detecting said probe hybridized to said analyte as an indication of the presence or amount of said analyte in said sample.

25 37. The method of claim 36, wherein said first nucleotide region of said probe includes one or more clusters of at least about 4 modified nucleotides.

38. The method of claim 36, wherein said first nucleotide region of said probe includes one or more
30 clusters of at least about 6 modified nucleotides.

39. The method of claim 36, wherein said first nucleotide region of said probe includes one or more clusters of at least about 8 modified nucleotides.

40. The method of claim 36, wherein substantially all of the nucleotides contained in said first nucleotide region of said probe are modified.

41. The method of claim 37, wherein at least one of said modified nucleotides includes a modification selected from the group consisting of:

- a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;
- c) a modification to the phosphate moiety;
- 10 d) a modification to the internucleoside linkage;
- and
- e) a modification to the internucleotide linkage.

42. The method of claim 37, wherein at least one of said modified nucleotides includes two modifications selected from the group consisting of:

- a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;
- c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage;
- 20 and
- e) a modification to the internucleotide linkage.

43. The method of claim 37, wherein at least one of said modified nucleotides includes a 2'-modification to the ribofuranosyl moiety selected from the group consisting of:

- a) an alkyl substitution;
- b) an alkoxy substitution; and
- c) a halide substitution.

44. The method of claim 37, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

45. The method of claim 37, wherein at least one of said modified nucleotides includes a propyne substitution to the nitrogenous base.

46. The method of claim 45, wherein said propyne
5 substitution is to a cytidine analog.

47. The method of claim 45, wherein said propyne substitution is to a thymidine analog.

48. The method of claim 37, wherein each said modified nucleotide of one or more of said clusters contains
10 the same modification.

49. The method of claim 48, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

50. The method of claim 36, wherein said probe
15 includes one or more conjugate molecules.

51. The method of claim 37, wherein said probe includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to said probe at a site located within one or more of said clusters contained in said first nucleotide region of said
20 probe.

52. The method of claim 37, wherein said probe is an oligonucleotide consisting of from about 10 to about 100 nucleotide bases.

25 53. The method of claim 37, wherein said probe is an oligonucleotide consisting of from about 10 to about 15 nucleotide bases.

54. The method of claim 37, wherein said probe is an oligonucleotide consisting of from about 12 to about 15 nucleotide bases.

55. The method of claim 37, wherein said probe
5 further includes a label.

56. The method of claim 55, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b) an enzyme,
- 10 c) an enzyme cofactor,
- d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- 15 h) a fluorescent molecule,
- i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- l) a nucleotide base sequence region that is unable
20 to stably hybridize to said analyte under said conditions.

57. The method of claim 56, wherein said label is a chemiluminescent molecule.

58. The method of claim 57, wherein light is emitted
by said chemiluminescent molecule upon the return to
25 ground state of an electronically excited N-acridone.

59. The method of claim 57, wherein said chemiluminescent molecule is an acridinium ester derivative.

60. The method of claim 55, wherein said label is
joined to said probe at a site located within one of said
30 clusters contained in said first nucleotide region of said
probe.

61. The method of claim 37, wherein said analyte consists of RNA.

62. The method of claim 61, wherein said RNA is rRNA or tRNA.

5 63. The method of claim 61, wherein said sample further contains DNA.

64. The method of claim 37, wherein said analyte consists of DNA.

65. The method of either claim 37 or 55, wherein
10 said probe or said analyte is directly or indirectly immobilized by a solid support.

66. The method of claim 36, wherein said contacting step further includes contacting said probe with a third nucleic acid, wherein said third nucleic acid contains a
15 first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said probe under selective hybridization conditions,

wherein said analyte is unable to stably hybridize to either said third nucleic acid or said second nucleotide
20 region of said probe under said conditions, and

wherein said third nucleic acid is unable to stably hybridize to said first nucleotide region of said probe under said conditions.

67. The method of claim 66, wherein at least one of
25 said nucleotide regions of said probe and said third nucleic acid includes one or more clusters of at least about 4 modified nucleotides.

68. The method of claim 66, wherein substantially all of the nucleotides contained in at least one of said

nucleotide regions of said probe and said third nucleic acid are modified.

69. The method of claim 67, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

70. The method of claim 67, wherein each said modified nucleotide of one or more of said clusters of at least one of said nucleotide regions of said probe and said third nucleic acid contains the same modification.

71. The method of claim 70, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

72. The method of claim 66, wherein at least one of said probe and said third nucleic acid includes one or more conjugate molecules.

73. The method of claim 67, wherein at least one of said probe and said third nucleic acid includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said probe and said third nucleic acid at a site located within one or more of said clusters contained in at least one of said nucleotide regions of said probe and said third nucleic acid.

74. The method of claim 67, wherein said probe further includes a label.

75. The method of claim 74, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b) an enzyme,
- c) an enzyme cofactor,

- d) an enzyme substrate,
 - e) a dye,
 - f) a hapten,
 - g) a chemiluminescent molecule,
 - 5 h) a fluorescent molecule,
 - i) a phosphorescent molecule,
 - j) an electrochemiluminescent molecule,
 - k) a chromophore, and
 - l) a nucleotide base sequence region that is unable
- 10 to stably hybridize to either said analyte or said third nucleic acid under said conditions.

76. The method of claim 75, wherein said label is a chemiluminescent molecule.

15 77. The method of claim 76, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

78. The method of claim 76, wherein said label is an acridinium ester derivative.

20 79. The method of claim 67, wherein said label is joined to said probe at a site located within one of said clusters contained in said first nucleotide region of said probe.

80. The method of claim 67, wherein said analyte consists of RNA.

25 81. The method of either claim 67 or 74, wherein one of said analyte and said third nucleic acid is directly or indirectly immobilized by a solid support.

82. The method of claim 36, wherein said contacting step further includes contacting said analyte with a third
30 nucleic acid, wherein said third nucleic acid contains a

first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under selective hybridization conditions,

wherein said probe is unable to stably hybridize to
5 either said third nucleic acid or said second nucleotide region of said analyte under said conditions, and

wherein said third nucleic acid is unable to stably hybridize to said first nucleotide region of said analyte under said conditions.

10 83. The method of claim 82, wherein at least one of said nucleotide regions of said probe and said third nucleic acid includes one or more clusters of at least about 4 modified nucleotides.

84. The method of claim 82, wherein substantially
15 all of the nucleotides contained in at least one of said nucleotide regions of said probe and said third nucleic acid are modified.

85. The method of claim 83, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.
20

86. The method of claim 83, wherein each said modified nucleotide of one or more of said clusters of at least one of said nucleotide regions of said probe and said third nucleic acid contains the same modification.

25 87. The method of claim 86, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

88. The method of claim 82, wherein at least one of said probe and said third nucleic acid includes one or
30 more conjugate molecules.

89. The method of claim 83, wherein at least one of said probe and said third nucleic acid includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said probe and said third nucleic acid at a site located within one or more of said clusters contained in at least one of said nucleotide regions of said probe and said third nucleic acid.

90. The method of claim 83, wherein said third nucleic acid is a helper probe.

91. The method of claim 83, wherein said probe further includes a label.

92. The method of claim 91, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b) an enzyme,
- c) an enzyme cofactor,
- d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- h) a fluorescent molecule,
- i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- l) a nucleotide base sequence region that is unable to stably hybridize to either said analyte or said third nucleic acid under said conditions.

93. The method of claim 92, wherein said label is a chemiluminescent molecule.

93

94. The method of claim 93, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

95. The method of claim 93, wherein said label is an
5 acridinium ester derivative.

96. The method of claim 91, wherein said label is joined to said probe at a site located within one of said clusters contained in said first nucleotide region of said probe.

10 97. The method of claim 83, wherein said analyte consists of RNA.

98. The method of either claim 83 or 91, wherein one of said probe and said third nucleic acid is directly or indirectly immobilized by a solid support.

15 99. The method of claim 36, wherein said contacting step further includes contacting a third nucleic acid with said analyte and said probe, wherein said third nucleic acid contains:

a) a first nucleotide base sequence region able to
20 stably hybridize to a second nucleotide base sequence region of said analyte under selective hybridization conditions; and

b) a second nucleotide base sequence region able to
25 stably hybridize to a second nucleotide base sequence region of said probe under selective hybridization conditions,

wherein said analyte is not able to stably hybridize to either said second nucleotide region of said probe or said second nucleotide region of said third nucleic acid
30 under said conditions,

wherein said probe is unable to stably hybridize to either said second nucleotide region of said analyte or

said first nucleotide region of said third nucleic acid under said conditions, and

wherein said third nucleic acid is unable to stably hybridize to either said first nucleotide region of said
5 analyte or said first nucleotide region of said probe.

100. The method of claim 99, wherein at least one of said nucleotide regions of said probe and said third nucleic acid includes one or more clusters of at least about 4 modified nucleotides.

10 101. The method of claim 99, wherein substantially all of the nucleotides contained in at least one of said nucleotide regions of said probe and said third nucleic acid are modified.

15 102. The method of claim 100, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

103. The method of claim 100, wherein each said modified nucleotide of one or more of said clusters of at least one of said nucleotide regions of said probe and
20 said third nucleic acid contains the same modification.

104. The method of claim 103, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

105. The method of claim 99, wherein at least one of
25 said probe and said third nucleic acid includes one or more conjugate molecules.

106. The method of claim 100, wherein at least one of said probe and said third nucleic acid includes one or more conjugate molecules, and wherein at least one of said
30 conjugate molecules is joined to at least one of said

probe and said third nucleic acid at a site located within one or more of said clusters contained in at least one of said nucleotide regions of said probe and said third nucleic acid.

5 107. The method of claim 100, wherein said probe further includes a label.

108. The method of claim 107, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- 10 b) an enzyme,
- c) an enzyme cofactor,
- d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- 15 g) a chemiluminescent molecule,
- h) a fluorescent molecule,
- i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- 20 l) a nucleotide base sequence region that is unable to stably hybridize to either said analyte or said third nucleic acid under said conditions.

109. The method of claim 108, wherein said label is a chemiluminescent molecule.

25 110. The method of claim 109, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

111. The method of claim 109, wherein said label is an acridinium ester derivative.

30 112. The method of claim 100, wherein said analyte consists of RNA.

113. The method of either claim 100 or 107, wherein one of said probe and said third nucleic acid is directly or indirectly immobilized by a solid support.

114. The method of claim 36, wherein said contacting
5 step further includes contacting the following components:

a) said probe with a third nucleic acid, wherein said third nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said probe under
10 selective hybridization conditions; and

b) said third nucleic acid with a fourth nucleic acid, wherein said fourth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said third
15 nucleic acid under selective hybridization conditions,

wherein said analyte is unable to stably hybridize to any of said second nucleotide region of said probe and said third and fourth nucleic acids under said conditions,

wherein said probe is unable to stably hybridize to
20 either of said second nucleotide region of said third nucleic acid and said fourth nucleic acid under said conditions,

wherein said third nucleic acid is unable to stably hybridize to said first nucleotide region of said probe
25 under said conditions, and

wherein said fourth nucleic acid is unable to stably hybridize to said first nucleotide region of said third nucleic acid under said conditions.

115. The method of claim 114, wherein at least one of
30 said nucleotide regions of said probe and said third and fourth nucleic acids includes one or more clusters of at least about 4 modified nucleotides.

116. The method of claim 114, wherein substantially all of the nucleotides contained in at least one of said

nucleotide regions of said probe and said third and fourth nucleic acids are modified.

117. The method of claim 115, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

118. The method of claim 115, wherein each said modified nucleotide of one or more of said clusters of at least one of said probe and said third and fourth nucleic acids contains the same modification.

119. The method of claim 118, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

120. The method of claim 114, wherein at least one of said probe and said third and fourth nucleic acids includes one or more conjugate molecules.

121. The method of claim 115, wherein at least one of said probe and said third and fourth nucleic acids includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said probe and said third and fourth nucleic acids at a site located within one or more of said clusters contained in at least one of said nucleotide regions of said probe and said third and fourth nucleic acids.

122. The method of claim 115, wherein said probe further includes a label.

123. The method of claim 122, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b) an enzyme,
- c) an enzyme cofactor,

- d) an enzyme substrate,
 - e) a dye,
 - f) a hapten,
 - g) a chemiluminescent molecule,
 - 5 h) a fluorescent molecule,
 - i) a phosphorescent molecule,
 - j) an electrochemiluminescent molecule,
 - k) a chromophore, and
 - l) a nucleotide base sequence region that is unable
- 10 to stably hybridize to any of said analyte and said third and fourth nucleic acids under said conditions.

124. The method of claim 123, wherein said label is a chemiluminescent molecule.

125. The method of claim 124, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

126. The method of claim 124, wherein said label is an acridinium ester derivative.

127. The method of claim 122, wherein said label is joined to said probe at a site located within one of said clusters of said first nucleotide region of said probe.

128. The method of claim 115, wherein said analyte consists of RNA.

129. The method of either claim 115 or 122, wherein said fourth nucleic acid is immobilized by a solid support.

130. The method of claim 36, wherein said contacting step further includes contacting the following components:

- a) said analyte with a third nucleic acid, wherein
- 30 said third nucleic acid contains a first nucleotide base

sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under selective hybridization conditions; and

5 b) said third nucleic acid with a fourth nucleic acid, wherein said fourth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said third nucleic acid under selective hybridization conditions,

10 wherein said analyte is unable to stably hybridize to either said second nucleotide region of said third nucleic acid or said fourth nucleic acid under said conditions,

 wherein said probe is unable to stably hybridize to any of said second nucleotide region of said analyte and said third and fourth nucleic acids under said conditions,

15 wherein said third nucleic acid is unable to stably hybridize to either said first nucleotide region of said analyte or said probe under said conditions, and

 wherein said fourth nucleic acid is unable to stably hybridize to said first nucleotide region of said third
20 nucleic acid under said conditions.

131. The method of claim 130, wherein at least one of said nucleotide regions of said probe and said third and fourth nucleic acids includes one or more clusters of at least about 4 modified nucleotides.

25 132. The method of claim 130, wherein substantially all of the nucleotides contained in at least one of said nucleotide regions of said probe and said third and fourth nucleic acids are modified.

30 133. The method of claim 131, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

134. The method of claim 131, wherein each said modified nucleotide of one or more of said clusters of at

least one of said nucleotide regions of said probe and said third and fourth nucleic acids contains the same modification.

135. The method of claim 134, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

136. The method of claim 130, wherein at least one of said probe and said third and fourth nucleic acids includes one or more conjugate molecules.

10 137. The method of claim 131, wherein at least one of said probe and said third and fourth nucleic acids includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said probe and said third and fourth nucleic
15 acids at a site located within one or more of said clusters contained in at least one of said nucleotide regions of said probe and said third and fourth nucleic acids.

138. The method of claim 131, wherein said probe further includes a label.

20 139. The method of claim 138, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b). an enzyme,
- c) an enzyme cofactor,
- 25 d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- h) a fluorescent molecule,
- 30 i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and

1) a nucleotide base sequence region that is unable to stably hybridize to any of said analyte and said third and fourth nucleic acids under said conditions.

140. The method of claim 139, wherein said label is
5 a chemiluminescent molecule.

141. The method of claim 140, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

142. The method of claim 140, wherein said label is
10 an acridinium ester derivative.

143. The method of claim 138, wherein said label is joined to said probe at a site located within one of said clusters of said first nucleotide region of said probe.

144. The method of claim 131, wherein said analyte
15 consists of RNA.

145. The method of either claim 131 or 138, wherein said fourth nucleic acid is immobilized by a solid support.

146. The method of claim 36, wherein said contacting
20 step further includes contacting the following components:

a) said analyte with a third nucleic acid, wherein said third nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under
25 selective hybridization conditions;

b) said probe with said third nucleic acid, wherein said third nucleic acid contains a second nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said probe under
30 selective hybridization conditions; and

c) said third nucleic acid with a fourth nucleic acid, wherein said fourth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a third nucleotide base sequence region of said third nucleic acid under selective hybridization conditions,

5 wherein said analyte is unable to stably hybridize to any of said second nucleotide region of said probe, said second and third nucleotide regions of said third nucleic acid, and said fourth nucleic acid under said conditions,

10 wherein said probe is unable to stably hybridize to any of said second nucleotide region of said analyte, said first and third nucleotide regions of said third nucleic acid, and said fourth nucleic acid under said conditions,

15 wherein said third nucleic acid is unable to stably hybridize to either said first nucleotide region of said analyte or said first nucleotide region of said probe under said conditions, and

20 wherein said fourth nucleic acid is unable to stably hybridize to either said first or second nucleotide region of said third nucleic acid under said conditions.

147. The method of claim 146, wherein at least one of said nucleotide regions of said probe and said third and fourth nucleic acids includes one or more clusters of at least about 4 modified nucleotides.

25 148. The method of claim 146, wherein substantially all of the nucleotides contained in at least one of said nucleotide regions of said probe and said third and fourth nucleic acids are modified.

30 149. The method of claim 147, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

150. The method of claim 147, wherein each said modified nucleotide of one or more of said clusters of at

least one of said nucleotide regions of said probe and said third and fourth nucleic acids contains the same modification.

151. The method of claim 150, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

152. The method of claim 146, wherein at least one of said probe and said third and fourth nucleic acids includes one or more conjugate molecules.

153. The method of claim 147, wherein at least one of said probe and said third and fourth nucleic acids includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said probe and said third and fourth nucleic acids at a site located within one or more of said clusters contained in at least one of said nucleotide regions of said probe and said third and fourth nucleic acids.

154. The method of claim 147, wherein said probe further includes a label.

155. The method of claim 154, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b) an enzyme,
- c) an enzyme cofactor,
- d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- h) a fluorescent molecule,
- i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and

1) a nucleotide base sequence region that is unable to stably hybridize to any of said analyte and said third and fourth nucleic acids under said conditions.

156. The method of claim 155, wherein said label is
5 a chemiluminescent molecule.

157. The method of claim 156, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

158. The method of claim 156, wherein said label is
10 an acridinium ester derivative.

159. The method of claim 154, wherein said label is joined to said probe at a site located within one of said clusters of said first nucleotide region of said probe.

160. The method of claim 147, wherein said analyte
15 consists of RNA.

161. The method of either claim 147 or 154, wherein said fourth nucleic acid is immobilized by a solid support.

162. The method of claim 36, wherein said contacting
20 step further includes contacting the following components:

a) said probe with a first coupling nucleic acid comprising a third nucleic acid, wherein said first coupling nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second
25 nucleotide base sequence region of said probe under selective hybridization conditions;

b) a second coupling nucleic acid comprising a fourth nucleic acid with a fifth nucleic acid, wherein said fifth nucleic acid contains a first nucleotide base
30 sequence region able to stably hybridize to a first

nucleotide base sequence region of said second coupling nucleic acid under selective hybridization conditions; and, alternatively,

5 i) said first coupling nucleic acid with said second coupling nucleic acid, wherein said second coupling nucleic acid contains a second nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said first coupling nucleic acid under selective hybridization conditions; or

10 ii) one or more optional coupling nucleic acids, other than said first and second coupling nucleic acids, wherein each of said optional coupling nucleic acids can stably hybridize to at least two other coupling nucleic acids under selective hybridization conditions, wherein at
15 least one of said other coupling nucleic acids may be at least one of said first and second coupling nucleic acids, and wherein each of said other coupling nucleic acids is directly or indirectly joined to said fifth nucleic acid and said analyte,

20 wherein said analyte is unable to stably hybridize to any of said second nucleotide region of said probe, said first and second coupling nucleic acids, said fifth nucleic acid, and said optional coupling nucleic acids under said conditions,

25 wherein said probe is unable to stably hybridize to any of said second nucleotide region of said first coupling nucleic acid, said second coupling nucleic acid, said fifth nucleic acid, and said optional coupling nucleic acids under said conditions,

30 wherein said first coupling nucleic acid is unable to stably hybridize to any of said first nucleotide region of said probe, said first nucleotide region of said second coupling nucleic acid, and said fifth nucleic acid under said conditions,

35 wherein said second coupling nucleic acid is unable to stably hybridize to said first nucleotide region of said first coupling nucleic acid under said conditions,

wherein said fifth nucleic acid is unable to stably hybridize to either said second nucleotide region of said second coupling nucleic acid or said optional coupling nucleic acids under said conditions, and

- 5 wherein said optional coupling nucleic acids do not stably hybridize to said first nucleotide region of either said first or second coupling nucleic acid under said conditions.

163. The method of claim 162, wherein at least one of
10 said nucleotide regions of said probe, said first and second coupling nucleic acids, said fifth nucleic acid, and a nucleotide base sequence region of any one of said optional coupling nucleic acids includes one or more clusters of at least about 4 modified nucleotides.

15 164. The method of claim 162, wherein substantially all of the nucleotides contained in at least one of said nucleotide regions of said probe, said first and second coupling nucleic acids, said fifth nucleic acid, and a nucleotide base sequence region of any one of said
20 optional coupling nucleic acids are modified.

165. The method of claim 163, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

166. The method of claim 163, wherein each said
25 modified nucleotide of one or more of said clusters of at least one of said nucleotide regions of said probe, said fifth nucleic acid, and any of said coupling nucleic acids contains the same modification.

167. The method of claim 166, wherein said modification
30 consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

168. The method of claim 162, wherein at least one of said probe, said fifth nucleic acid, and any of said coupling nucleic acids includes one or more conjugate molecules.

5 169. The method of claim 163, wherein at least one of said probe, said fifth nucleic acid, and any of said coupling nucleic acids includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said probe, said
10 fifth nucleic acid, and any of said coupling nucleic acids at a site located within one or more of said clusters contained in at least one of said nucleotide regions of said probe, said fifth nucleic acid, and any of said coupling nucleic acids.

15 170. The method of claim 163, wherein said probe further includes a label.

171. The method of claim 170, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- 20 b) an enzyme,
- c) an enzyme cofactor,
- d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- 25 g) a chemiluminescent molecule,
- h) a fluorescent molecule,
- i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- 30 l) a nucleotide base sequence region that is unable to stably hybridize to any of said analyte, said fifth nucleic acid, and said coupling nucleic acids under said conditions.

172. The method of claim 171, wherein said label is a chemiluminescent molecule.

173. The method of claim 172, wherein light is emitted by said chemiluminescent molecule upon the return to
5 ground state of an electronically excited N-acridone.

174. The method of claim 172, wherein said label is an acridinium ester derivative.

175. The method of claim 170, wherein said label is joined to said probe at a site located within one of said
10 clusters contained in said first nucleotide region of said probe.

176. The method of claim 163, wherein said analyte consists of RNA.

177. The method of either claim 163 or 170, wherein
15 said fifth nucleic acid is immobilized by a solid support.

178. The method of claim 36, wherein said contacting step further includes contacting the following components:

a) said analyte with a first coupling nucleic acid comprising a third nucleic acid, wherein said first coupling
20 nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under selective hybridization conditions;

b) a second coupling nucleic acid comprising a
25 fourth nucleic acid with a fifth nucleic acid, wherein said fifth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said second coupling nucleic acid under selective hybridization conditions;
30 and, alternatively,

i) said first coupling nucleic acid with said second coupling nucleic acid, wherein said second coupling nucleic acid contains a second nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said first coupling nucleic acid under selective hybridization conditions; or

ii) one or more optional coupling nucleic acids, other than said first and second coupling nucleic acids, wherein each of said optional coupling nucleic acids is able to stably hybridize to two other coupling nucleic acids under selective hybridization conditions, wherein at least one of said other coupling nucleic acids may be at least one of said first and second coupling nucleic acids, and wherein each of said other coupling nucleic acids is directly or indirectly joined to said fifth nucleic acid and said analyte,

wherein said analyte is unable to stably hybridize to any of said second nucleotide region of said first coupling nucleic acid, said second coupling nucleic acid, said fifth nucleic acid, and said optional coupling nucleic acids under said conditions,

wherein said probe is unable to stably hybridize to any of said second nucleotide region of said analyte, said first and second coupling nucleic acids, said fifth nucleic acid, and said optional coupling nucleic acids under said conditions,

wherein said first coupling nucleic acid is unable to stably hybridize to any of said first nucleotide region of said analyte, said first nucleotide region of said second coupling nucleic acid, and said fifth nucleic acid under said conditions,

wherein said second coupling nucleic acid is unable to stably hybridize to said first nucleotide region of said first coupling nucleic acid under said conditions,

wherein said fifth nucleic acid is unable to stably hybridize to either said second nucleotide region of said

110

second coupling nucleic acid or said optional coupling nucleic acids under said conditions, and

wherein said optional coupling nucleic acids do not stably hybridize to said first nucleotide region of either
5 said first or second coupling nucleic acid under said conditions.

179. The method of claim 178, wherein at least one of said nucleotide regions of said probe, said first and second coupling nucleic acids, said fifth nucleic acid,
10 and a nucleotide base sequence region of any one of said optional coupling nucleic acids includes one or more clusters of at least about 4 modified nucleotides.

180. The method of claim 178, wherein substantially all of the nucleotides contained in at least one of said
15 nucleotide regions of said probe, said first and second coupling nucleic acids, said fifth nucleic acid, and a nucleotide base sequence region of any one of said optional coupling nucleic acids are modified.

181. The method of claim 179, wherein at least one of
20 said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

182. The method of claim 179, wherein each said modified nucleotide of one or more of said clusters of at least one of said nucleotide regions of said probe, said
25 fifth nucleic acid, and any of said coupling nucleic acids contains the same modification.

183. The method of claim 182, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

30 184. The method of claim 178, wherein at least one of said probe, said fifth nucleic acid, and any of said

111

coupling nucleic acids includes one or more conjugate molecules.

185. The method of claim 179, wherein at least one of said probe, said fifth nucleic acid, and any of said
5 coupling nucleic acids includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said probe, said fifth nucleic acid, and any of said coupling nucleic acids
10 at a site located within one or more of said clusters contained in at least one of said nucleotide regions of said probe, said fifth nucleic acid, and any of said coupling nucleic acids.

186. The method of claim 179, wherein said probe further includes a label.

15 187. The method of claim 186, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b) an enzyme,
- c) an enzyme cofactor,
- 20 d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- h) a fluorescent molecule,
- 25 i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- l) a nucleotide base sequence region that is unable
to stably hybridize to any of said analyte, said fifth
30 nucleic acid, and any of said coupling nucleic acids under said conditions.

188. The method of claim 187, wherein said label is a chemiluminescent molecule.

189. The method of claim 188, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

190. The method of claim 188, wherein said label is
5 an acridinium ester derivative.

191. The method of claim 186, wherein said label is joined to said probe at a site located within said first nucleotide region of said probe.

192. The method of claim 179, wherein said analyte
10 consists of RNA.

193. The method of either claim 179 or 186, wherein said fifth nucleic acid is immobilized by a solid support.

194. The method of claim 36, wherein said contacting step further includes contacting the following components:
15 a) said probe with a first coupling nucleic acid comprising a third nucleic acid, wherein said first coupling nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said probe under
20 selective hybridization conditions;

b) said analyte with said first coupling nucleic acid, wherein said first coupling nucleic acid contains a second nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of
25 said analyte under selective hybridization conditions;

c) a second coupling nucleic acid comprising a fourth nucleic acid with a fifth nucleic acid, wherein said fifth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a first
30 nucleotide base sequence region of said second coupling nucleic acid under selective hybridization conditions; and, alternatively,

113

i) said first coupling nucleic acid with said second coupling nucleic acid, wherein said second coupling nucleic acid contains a second nucleotide base sequence region able to stably hybridize to a third nucleotide base sequence region of said first coupling nucleic acid under selective hybridization conditions; or

ii) one or more optional coupling nucleic acids, other than said first and second coupling nucleic acids, wherein each of said optional coupling nucleic acids is able to stably hybridize to two other coupling nucleic acids under selective hybridization conditions, wherein at least one of said other coupling nucleic acids may be at least one of said first and second coupling nucleic acids, and wherein each of said other coupling nucleic acids is directly or indirectly joined to said analyte, said probe and said fifth nucleic acid,

wherein said analyte is unable to stably hybridize to any of said second nucleotide region of said probe, said first and third nucleotide regions of said first coupling nucleic acid, said second coupling nucleic acid, said fifth nucleic acid, and said optional coupling nucleic acids under said conditions,

wherein said probe is unable to stably hybridize to any of said second nucleotide region of said analyte, said second and third nucleotide regions of said first coupling nucleic acid, said second coupling nucleic acid, said fifth nucleic acid, and said optional coupling nucleic acids under said conditions,

wherein said first coupling nucleic acid is unable to stably hybridize to any of said first nucleotide region of said analyte, said first nucleotide region of said probe, said first nucleotide region of said second coupling nucleic acid, and said fifth nucleic acid under said conditions,

wherein said second coupling nucleic acid is unable to stably hybridize to either said first or second nucleo-

tide region of said first coupling nucleic acid under said conditions,

wherein said fifth nucleic acid is unable to stably hybridize to either said second nucleotide region of said
5 second coupling nucleic acid or said optional coupling nucleic acids under said conditions, and

wherein said optional coupling nucleic acids do not stably hybridize to any of said first and second nucleotide regions of said first coupling nucleic acid and said
10 first nucleotide region of said second coupling nucleic acid under said conditions.

195. The method of claim 194, wherein at least one of said nucleotide regions of said probe, said first and second coupling nucleic acids, said fifth nucleic acid,
15 and a nucleotide base sequence region of any of said optional coupling nucleic acids includes one or more clusters of at least about 4 modified nucleotides.

196. The method of claim 194, wherein substantially all of the nucleotides contained in at least one of said
20 nucleotide regions of said probe, said first and second coupling nucleic acids, said fifth nucleic acid, and a nucleotide base sequence region of any of said optional coupling nucleic acids are modified.

197. The method of claim 195, wherein at least one of
25 said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

198. The method of claim 195, wherein each said modified nucleotide of one or more of said clusters of at least one of said nucleotide regions of said probe, said
30 fifth nucleic acid, and any of said coupling nucleic acids contains the same modification.

199. The method of claim 198, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

200. The method of claim 194, wherein at least one of
5 said probe, said fifth nucleic acid, and any of said coupling nucleic acids includes one or more conjugate molecules.

201. The method of claim 195, wherein at least one of
said probe, said fifth nucleic acid, and any of said
10 coupling nucleic acids includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said probe, said fifth nucleic acid, and any of said coupling nucleic acids
15 at a site located within one or more of said clusters contained in at least one of said nucleotide regions of said probe, said fifth nucleic acid, and any of said coupling nucleic acids.

202. The method of claim 195, wherein said probe further includes a label.

20 203. The method of claim 202, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b) an enzyme,
- c) an enzyme cofactor,
- 25 d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- h) a fluorescent molecule,
- 30 i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and

1) a nucleotide base sequence region that is unable to stably hybridize to any of said analyte, said fifth nucleic acid, and said coupling nucleic acids under said conditions.

5 204. The method of claim 203, wherein said label is a chemiluminescent molecule.

205. The method of claim 204, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

10 206. The method of claim 204, wherein said label is an acridinium ester derivative.

207. The method of claim 202, wherein said label is joined to said probe at a site located within said first nucleotide region of said probe.

15 208. The method of claim 195, wherein said analyte consists of RNA.

209. The method of either claim 195 or 202, wherein said fifth nucleic acid is immobilized by a solid support.

20 210. A method for detecting the presence or amount of an analyte comprising a first nucleic acid in a sample suspected of containing said analyte, which comprises the steps of:

a) contacting the following components:

25 i) said sample with a second nucleic acid, wherein said second nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said analyte under selective hybridization conditions; and

30 ii) a probe comprising a third nucleic acid with said second nucleic acid, wherein said second nucleic acid

contains a second nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said probe under selective hybridization conditions,

5 wherein said second nucleic acid cannot stably hybridize to said probe unless said second nucleic acid is stably hybridized with said analyte,

 wherein said analyte is unable to stably hybridize to either said probe or said second nucleotide region of said
10 second nucleic acid under said conditions,

 wherein said probe is unable to stably hybridize to said first nucleotide region of said second nucleic acid under said conditions,

 wherein said second nucleic acid is unable to stably
15 hybridize to said first nucleotide region of said analyte under said conditions, and

 wherein at least one of said nucleotide regions of said probe and said second nucleic acid includes one or more modified nucleotides;

20 b) subjecting the components of step a) to selective hybridization conditions, so that

 if at least one of said first nucleotide region of said probe and said second nucleotide region of said
25 second nucleic acid includes one or more of said modified nucleotides, then

 i) the hybridization binding affinity between said probe and said second nucleic acid is greater than the hybridization binding affinity between unmodified forms of
30 said probe and said second nucleic acid, under identical hybridization conditions, and

 ii) the hybridization rate between said probe and said second nucleic acid is greater than the hybridization rate between unmodified forms of said probe and said
35 second nucleic acid, under identical hybridization conditions, and

if said first nucleotide region of said second nucleic acid includes one or more of said modified nucleotides, then

i) the hybridization binding affinity between said
5 analyte and said second nucleic acid is greater than the hybridization binding affinity between said analyte and an unmodified form of said second nucleic acid, under identical hybridization conditions, and

ii) the hybridization rate between said analyte and
10 said second nucleic acid is greater than the hybridization rate between said analyte and an unmodified form of said second nucleic acid, under identical hybridization conditions; and

c) detecting said labeled probe hybridized with
15 said second nucleic acid as an indication of the presence or amount of said analyte in said sample.

211. The method of claim 210, wherein at least one of said nucleotide regions of said probe and said second nucleic acid includes one or more clusters of at least
20 about 4 modified nucleotides.

212. The method of claim 210, wherein at least one of said nucleotide regions of said probe and said second nucleic acid includes one or more clusters of at least about 6 modified nucleotides.

25 213. The method of claim 210, wherein at least one of said nucleotide regions of said probe and said second nucleic acid includes one or more clusters of at least about 8 modified nucleotides.

214. The method of claim 210, wherein substantially
30 all of the nucleotides contained in at least one of said nucleotide regions of said probe and said second nucleic acid are modified.

215. The method of claim 211, wherein at least one of said modified nucleotides includes a modification selected from the group consisting of:

- a) a modification to the nitrogenous base;
- 5 b) a modification to the sugar moiety;
- c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage;
- and
- e) a modification to the internucleotide linkage.

10 216. The method of claim 211, wherein at least one of said modified nucleotides includes two modifications selected from the group consisting of:

- a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;
- 15 c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage;
- and
- e) a modification to the internucleotide linkage.

217. The method of claim 211, wherein at least one of
20 said modified nucleotides includes a 2'-modification to the ribofuranosyl moiety selected from the group consisting of:

- a) an alkyl substitution;
- b) an alkoxy substitution; and
- 25 c) a halide substitution.

218. The method of claim 211, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

219. The method of claim 211, wherein at least one of
30 said modified nucleotides includes a propyne substitution to the nitrogenous base.

120

220. The method of claim 219, wherein said propyne substitution is to a cytidine analog.

221. The method of claim 219, wherein said propyne substitution is to a thymidine analog.

5 222. The method of claim 211, wherein each said modified nucleotide of one or more of said clusters of at least one of said nucleotide regions of said probe and said second nucleic acid contains the same modification.

10 223. The method of claim 222, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

224. The method of claim 210, wherein at least one of said probe and said second nucleic acid includes one or more conjugate molecules.

15 225. The method of claim 211, wherein at least one of said probe and said second nucleic acid includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said probe and said second nucleic acid at a site located
20 within one or more of said clusters contained in at least one of said nucleotide regions of said probe and said second nucleic acid.

226. The method of claim 211, wherein said probe is an oligonucleotide consisting of from about 10 to about
25 100 nucleotide bases.

227. The method of claim 211, wherein said probe is an oligonucleotide consisting of from about 10 to about 15 nucleotide bases.

121

228. The method of claim 211, wherein said probe is an oligonucleotide consisting of from about 12 to about 15 nucleotide bases.

229. The method of claim 211, wherein said probe
5 further includes a label.

230. The method of claim 229, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b) an enzyme,
- 10 c) an enzyme cofactor,
- d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- 15 h) a fluorescent molecule,
- i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- l) a nucleotide base sequence region that is unable
20 to stably hybridize to either said analyte or said second nucleic acid under said conditions.

231. The method of claim 230, wherein said label is a chemiluminescent molecule.

232. The method of claim 231, wherein light is emitted
25 by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

233. The method of claim 231, wherein said chemiluminescent molecule is an acridinium ester derivative.

234. The method of claim 229, wherein said label is
30 joined to said probe at a site located within one of said

clusters contained in said first nucleotide region of said probe.

235. The method of claim 211, wherein said analyte consists of RNA.

5 236. The method of claim 235, wherein said RNA is rRNA or tRNA.

237. The method of claim 235, wherein said sample further contains DNA.

10 238. The method of claim 211, wherein said analyte consists of DNA.

239. The method of either claim 211 or 229, wherein at least one of said analyte, said probe and said second nucleic acid is directly or indirectly immobilized by a solid support.

15 240. A method for detecting the presence or amount of an analyte comprising a first nucleic acid in a sample suspected of containing said analyte, which comprises the steps of:

- a) contacting the following components:
 - 20 i) said sample with a probe comprising a second nucleic acid, wherein said probe contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said analyte under selective hybridization conditions;
 - 25 ii) said analyte with a third nucleic acid, wherein said third nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under selective hybridization conditions; and
 - 30 iii) said third nucleic acid with a fourth nucleic acid, wherein said fourth nucleic acid contains a first

nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said third nucleic acid under selective hybridization conditions,

wherein said third nucleic acid cannot stably
5 hybridize to said fourth nucleic acid unless said third nucleic acid is stably hybridized with said analyte,

wherein said analyte is unable to stably hybridize to either said second nucleotide region of said third nucleic acid or said fourth nucleic acid under said conditions,

10 wherein said probe is unable to stably hybridize any of said second nucleotide region of said analyte and said third and fourth nucleic acids under said conditions,

wherein said third nucleic acid is unable to stably hybridize to said first nucleotide region of said analyte
15 under said conditions,

wherein said fourth nucleic acid is unable to stably hybridize to said first nucleotide region of said third nucleic acid under said conditions, and

wherein at least one of said nucleotide regions of
20 said probe and said third and fourth nucleic acids includes one or more modified nucleotides;

b) subjecting the components of step (a) to selective hybridization conditions, so that

if said first nucleotide region of said probe
25 includes one or more of said modified nucleotides, then

i) the hybridization binding affinity between said analyte and said probe is greater than the hybridization binding affinity between said analyte and an unmodified form of said probe, under identical hybridization
30 conditions, and

ii) the hybridization rate between said analyte and said probe is greater than the hybridization rate between said analyte and an unmodified form of said probe, under identical hybridization conditions,

35 if said first nucleotide region of said third nucleic acid includes one or more of said modified nucleotides, then

i) the hybridization binding affinity between said analyte and said third nucleic acid is greater than the hybridization binding affinity between said analyte and an unmodified form of said third nucleic acid, under identical hybridization conditions, and

ii) the hybridization rate between said analyte and said third nucleic acid is greater than the hybridization rate between said analyte and an unmodified form of said third nucleic acid, under identical hybridization conditions,

if at least one of said second nucleotide region of said third nucleic acid and said first nucleotide region of said fourth nucleic acid includes one or more of said modified nucleotides, then

i) the hybridization binding affinity between said third and fourth nucleic acids is greater than the hybridization binding affinity between unmodified forms of said third and fourth nucleic acids, under identical hybridization conditions, and

ii) the hybridization rate between said third and fourth nucleic acids is greater than the hybridization rate between unmodified forms of said third and fourth nucleic acids, under identical hybridization conditions; and

c) detecting said labeled probe hybridized with said analyte, as an indication of the presence or amount of said analyte in said sample.

241. The method of claim 240, wherein at least one of said nucleotide regions of said probe and said third and fourth nucleic acids includes one or more clusters of at least about 4 modified nucleotides.

242. The method of claim 240, wherein at least one of said nucleotide regions of said probe and said third and fourth nucleic acids includes one or more clusters of at least about 6 modified nucleotides.

243. The method of claim 240, wherein at least one of said nucleotide regions of said probe and said third and fourth nucleic acids includes one or more clusters of at least about 8 modified nucleotides.

5 244. The method of claim 240, wherein substantially all of the nucleotides contained in at least one of said nucleotide regions of said probe and said third and fourth nucleic acids are modified.

10 245. The method of claim 241, wherein at least one of said modified nucleotides includes a modification selected from the group consisting of:

- a) a modification to the nitrogenous base;
 - b) a modification to the sugar moiety;
 - c) a modification to the phosphate moiety;
 - 15 d) a modification to the internucleoside linkage;
- and
- e) a modification to the internucleotide linkage.

246. The method of claim 241, wherein at least one of said modified nucleotides includes two modifications
20 selected from the group consisting of:

- a) a modification to the nitrogenous base;
 - b) a modification to the sugar moiety;
 - c) a modification to the phosphate moiety;
 - d) a modification to the internucleoside linkage;
 - 25 and
- e) a modification to the internucleotide linkage.

247. The method of claim 241, wherein at least one of said modified nucleotides includes a 2'-modification to the ribofuranosyl moiety selected from the group
30 consisting of:

- a) an alkyl substitution;
- b) an alkoxy substitution; and
- c) a halide substitution.

248. The method of claim 241, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

249. The method of claim 241, wherein at least one of
5 said modified nucleotides includes a propyne substitution to the nitrogenous base.

250. The method of claim 249, wherein said propyne substitution is to a cytidine analog.

251. The method of claim 249, wherein said propyne
10 substitution is to a thymidine analog.

252. The method of claim 241, wherein each said modified nucleotide of one or more of said clusters of at least one of said nucleotide regions of said probe and said third and fourth nucleic acids contains the same
15 modification.

253. The method of claim 252, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

254. The method of claim 240, wherein at least one of
20 said probe and said third nucleic acid includes one or more conjugate molecules.

255. The method of claim 241, wherein at least one of said probe and said third nucleic acid includes one or more conjugate molecules, and wherein at least one of said
25 conjugate molecules is joined to at least one of said probe and said third and fourth nucleic acids at a site located within one or more of said clusters contained in at least one of said nucleotide regions of said probe and said third and fourth nucleic acids.

256. The method of claim 241, wherein said probe is an oligonucleotide consisting of from about 10 to about 100 nucleotide bases.

257. The method of claim 241, wherein said probe is
5 an oligonucleotide consisting of from about 10 to about 15 nucleotide bases.

258. The method of claim 241, wherein said probe is an oligonucleotide consisting of from about 12 to about 15 nucleotide bases.

10 259. The method of claim 241, wherein said probe further includes a label.

260. The method of claim 259, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- 15 b) an enzyme,
- c) an enzyme cofactor,
- d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- 20 g) a chemiluminescent molecule,
- h) a fluorescent molecule,
- i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- 25 l) a nucleotide base sequence region that is unable to stably hybridize to any of said analyte and said third and fourth nucleic acids under said conditions.

261. The method of claim 260, wherein said label is a chemiluminescent molecule.

262. The method of claim 261, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

263. The method of claim 261, wherein said chemiluminescent molecule is an acridinium ester derivative.

264. The method of claim 259, wherein said label is joined to said probe at a site located within one of said clusters contained in said first nucleotide region of said probe.

265. The method of claim 241, wherein said analyte consists of RNA.

266. The method of claim 265, wherein said RNA is rRNA or tRNA.

267. The method of claim 265, wherein said sample further contains DNA.

268. The method of claim 241, wherein said analyte consists of DNA.

269. The method of either claim 241 or 259, wherein said fourth nucleic acid is immobilized by a solid support.

270. A method for detecting the presence or amount of an analyte comprising a first nucleic acid in a sample suspected of containing said analyte, which comprises the steps of:

- a) contacting the following components:
 - i) said sample with a second nucleic acid, wherein said second nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a first

nucleotide base sequence region of said analyte under selective hybridization conditions;

ii) said analyte with a probe comprising a third nucleic acid, wherein said probe contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under selective hybridization conditions; and

iii) said probe with said second nucleic acid, wherein said second nucleic acid contains a second nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said probe under selective hybridization conditions,

wherein said probe cannot stably hybridize to said analyte and said second nucleic acid unless said second nucleic acid is stably hybridized with said analyte,

wherein said analyte is unable to stably hybridize to either said second nucleotide region of said probe or said second nucleotide region of said second nucleic acid under said conditions,

wherein said probe is unable to stably hybridize to either said first nucleotide region of said analyte or said first nucleotide region of said second nucleic acid under said conditions,

wherein said second nucleic acid is unable to stably hybridize to either said second nucleotide region of said analyte or said first nucleotide region of said probe under said conditions, and

wherein at least one of said nucleotide regions of said probe and said second nucleic acid includes one or more modified nucleotides;

b) subjecting the components of step a) to selective hybridization conditions, so that

if said first nucleotide region of said probe includes one or more of said modified nucleotides, then

i) the hybridization binding affinity between said analyte and said probe is greater than the hybridization binding affinity between said analyte and an unmodified

form of said probe, under identical hybridization conditions, and

ii) the hybridization rate between said analyte and said probe is greater than the hybridization rate between
5 said analyte and an unmodified form of said probe, under identical hybridization conditions,

if said first nucleotide region of said second nucleic acid includes one or more of said modified nucleotides, then

10 i) the hybridization binding affinity between said analyte and said second nucleic acid is greater than the hybridization binding affinity between said analyte and an unmodified form of said second nucleic acid, under identical hybridization conditions, and

15 ii) the hybridization rate between said analyte and said second nucleic acid is greater than the hybridization rate between said analyte and an unmodified form of said second nucleic acid, under identical hybridization conditions, and

20 if at least one of said second nucleotide region of said probe and said second nucleotide region of said second nucleic acid includes one or more of said modified nucleotides, then

i) the hybridization binding affinity between said
25 probe and said second nucleic acid is greater than the hybridization binding affinity between unmodified forms of said probe and said second nucleic acid, under identical hybridization conditions, and

ii) the hybridization rate between said probe and
30 said second nucleic acid is greater than the hybridization rate between unmodified forms of said probe and said second nucleic acid, under identical hybridization conditions; and

c) detecting said labeled probe hybridized with
35 said analyte as an indication of the presence or amount of said analyte in said sample.

271. The method of claim 270, wherein at least one of said nucleotide regions of said probe and said second nucleic acid includes one or more clusters of at least about 4 modified nucleotides.

5 272. The method of claim 270, wherein at least one of said nucleotide regions of said probe and said second nucleic acid includes one or more clusters of at least about 6 modified nucleotides.

10 273. The method of claim 270, wherein at least one of said nucleotide regions of said probe and said second nucleic acid includes one or more clusters of at least about 8 modified nucleotides.

15 274. The method of claim 270, wherein substantially all of the nucleotides contained in at least one of said nucleotide regions of said probe and said second nucleic acid are modified.

275. The method of claim 271, wherein at least one of said modified nucleotides includes a modification selected from the group consisting of:

- 20 a) a modification to the nitrogenous base;
 b) a modification to the sugar moiety;
 c) a modification to the phosphate moiety;
 d) a modification to the internucleoside linkage;
 and
25 e) a modification to the internucleotide linkage.

276. The method of claim 271, wherein at least one of said modified nucleotides includes two modifications selected from the group consisting of:

- 30 a) a modification to the nitrogenous base;
 b) a modification to the sugar moiety;
 c) a modification to the phosphate moiety;

132

- d) a modification to the internucleoside linkage;
and
- e) a modification to the internucleotide linkage.

277. The method of claim 271, wherein at least one of
5 said modified nucleotides includes a 2'-modification to
the ribofuranosyl moiety selected from the group
consisting of:

- a) an alkyl substitution;
- b) an alkoxy substitution; and
- 10 c) a halide substitution.

278. The method of claim 271, wherein at least one of
said modified nucleotides includes a 2'-O-methyl
substitution to the ribofuranosyl moiety.

279. The method of claim 271, wherein at least one of
15 said modified nucleotides includes a propyne substitution
to the nitrogenous base.

280. The method of claim 279, wherein said propyne
substitution is to a cytidine analog.

281. The method of claim 279, wherein said propyne
20 substitution is to a thymidine analog.

282. The method of claim 271, wherein each said
modified nucleotide of one or more of said clusters of at
least one of said nucleotide regions of said probe and
said second nucleic acid contains the same modification.

283. The method of claim 282, wherein said
25 modification consists of a 2'-O-methyl substitution to the
ribofuranosyl moiety.

284. The method of claim 270, wherein at least one of said probe and said second nucleic acid includes one or more conjugate molecules.

285. The method of claim 271, wherein at least one of
5 said probe and said second nucleic acid includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to at least one of said probe and said second nucleic acid at a site located within one or more of said clusters contained in at least
10 one of said nucleotide regions of said probe and said second nucleic acid.

286. The method of claim 271, wherein said probe is an oligonucleotide consisting of from about 10 to about 100 nucleotide bases.

15 287. The method of claim 271, wherein said probe is an oligonucleotide consisting of from about 10 to about 15 nucleotide bases.

288. The method of claim 271, wherein said probe is an oligonucleotide consisting of from about 12 to about 15
20 nucleotide bases.

289. The method of claim 271, wherein said probe further includes a label.

290. The method of claim 289, wherein said label is selected from the group consisting of:

- 25 a) a radioisotope,
 b) an enzyme,
 c) an enzyme cofactor,
 d) an enzyme substrate,
 e) a dye,
30 f) a hapten,
 g) a chemiluminescent molecule,

- h) a fluorescent molecule,
- i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- 5 l) a nucleotide base sequence region that is unable to stably hybridize to either said analyte or said second nucleic acid under said conditions.

291. The method of claim 290, wherein said label is a chemiluminescent molecule.

- 10 292. The method of claim 291, wherein light is emitted by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

293. The method of claim 291, wherein said chemiluminescent molecule is an acridinium ester derivative.

- 15 294. The method of claim 289, wherein said label is joined to said probe at a site located within one of said clusters contained in said first nucleotide region of said probe.

- 20 295. The method of claim 271, wherein said analyte consists of RNA.

296. The method of claim 295, wherein said RNA is rRNA or tRNA.

297. The method of claim 295, wherein said sample further contains DNA.

- 25 298. The method of claim 271, wherein said analyte consists of DNA.

299. The method of claim either 271 or 289, wherein at least one of said analyte, said probe and said second

nucleic acid is directly or indirectly immobilized by a solid support.

300. A method for detecting the presence or amount of an analyte comprising a first nucleic acid in a sample suspected of containing said analyte, which comprises the steps of:

a) contacting the following components:

i) said sample with a probe comprising a second nucleic acid, wherein said probe contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said analyte under selective hybridization conditions;

ii) said probe with a third nucleic acid, wherein said third nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said probe under selective hybridization conditions; and

iii) said third nucleic acid with a fourth nucleic acid, wherein said fourth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said third nucleic acid under selective hybridization conditions,

wherein said analyte is unable to stably hybridize to any of said second nucleotide region of said probe and said third and fourth nucleic acids under said conditions,

wherein said probe is unable to stably hybridize to either said second nucleotide region of said third nucleic acid or said fourth nucleic acid under said conditions,

wherein said third nucleic acid is unable to stably hybridize to said first nucleotide region of said probe under said conditions,

wherein said fourth nucleic acid is unable to stably hybridize to said first nucleotide region of said third nucleic acid under said conditions, and

wherein said first nucleotide region of said third nucleic acid is unable to stably hybridize to said fourth nucleic acid under said conditions;

b) subjecting the components of step a) to
5 selective hybridization conditions; and

c) detecting said probe hybridized with said analyte as an indication of the presence or amount of said analyte in said sample.

301. The method of claim 300, wherein said probe
10 further includes a label.

302. The method of claim 301, wherein said label is joined to said probe at a site located within said first nucleotide region of said probe.

303. The method of either claim 300 or 301, wherein
15 said fourth nucleic acid is immobilized by a solid support.

304. A method for detecting the presence or amount of an analyte comprising a first nucleic acid in a sample suspected of containing said analyte, which comprises the
20 steps of:

a) contacting the following components:

i) said sample with a probe comprising a second nucleic acid, wherein said probe contains a first nucleotide base sequence region able to stably hybridize to a
25 first nucleotide base sequence region of said analyte under selective hybridization conditions;

ii) said analyte with a third nucleic acid, wherein said third nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second
30 nucleotide base sequence region of said analyte under selective hybridization conditions; and

iii) said third nucleic acid with a fourth nucleic acid, wherein said fourth nucleic acid contains a first

nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said third nucleic acid under selective hybridization conditions,

wherein said analyte is unable to stably hybridize to either said second nucleotide region of said third nucleic acid or said fourth nucleic acid under said conditions,

wherein said probe is unable to stably hybridize to any of said second nucleotide region of said analyte and said third and fourth nucleic acids under said conditions, and

wherein said first nucleotide region of said third nucleic acid is unable to stably hybridize to said fourth nucleic acid under said conditions;

b) subjecting the components of step a) to selective hybridization conditions; and

c) detecting said probe hybridized with said analyte as an indication of the presence or amount of said analyte in said sample.

305. The method of claim 304, wherein said probe further includes a label.

306. The method of claim 305, wherein said label is joined to said probe at a site located within said first nucleotide region of said probe.

307. The method of either claim 304 or 305, wherein said fourth nucleic acid is immobilized by a solid support.

308. A method for detecting the presence or amount of an analyte comprising a first nucleic acid in a sample suspected of containing said analyte, which comprises the steps of:

a) contacting the following components:

i) said sample with a probe comprising a second nucleic acid, wherein said probe contains a first nucleo-

tide base sequence region able to stably hybridize to a first nucleotide base sequence region of said analyte under selective hybridization conditions;

ii) said analyte with a third nucleic acid, wherein
5 said third nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under selective hybridization conditions;

iii) said probe with said third nucleic acid, wherein
10 said third nucleic acid contains a second nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said probe under selective hybridization conditions; and

iv) said third nucleic acid with a fourth nucleic
15 acid, wherein said fourth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a third nucleotide base sequence region of said third nucleic acid under selective hybridization conditions,

wherein said analyte is unable to stably hybridize to
20 any of said second nucleotide region of said probe, said second and third nucleotide regions of said third nucleic acid, and said fourth nucleic acid under said conditions,

wherein said probe is unable to stably hybridize to
any of said second nucleotide region of said analyte, said
25 first and third nucleotide regions of said third nucleic acid, and said fourth nucleic acid under said conditions, and

wherein said first and second nucleotide regions of
said third nucleic acid do not stably hybridize to said
30 fourth nucleic acid under said conditions;

b) subjecting the components of step a) to selective hybridization conditions; and

c) detecting said probe hybridized with said analyte as an indication of the presence or amount of said
35 analyte in said sample.

139

309. The method of claim 308, wherein said probe further includes a label.

310. The method of claim 309, wherein said label is joined to said probe at a site located within said first
5 nucleotide region of said probe.

311. The method of either claim 308 or 309, wherein said fourth nucleic acid is immobilized by a solid support.

312. A method for detecting the presence or amount of
10 an analyte comprising a first nucleic acid in a sample suspected of containing said sample, which comprises the steps of:

a) contacting the following components:

i) said sample with a probe comprising a second
15 nucleic acid, wherein said probe contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said analyte under selective hybridization conditions;

ii) said probe with a first coupling nucleic acid
20 comprising a third nucleic acid, wherein said first coupling nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said probe under selective hybridization conditions;

iii) a second coupling nucleic acid comprising a
25 fourth nucleic acid with a fifth nucleic acid, wherein said fifth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said second coupling
30 nucleic acid under selective hybridization conditions;
and, alternatively,

(a) said first coupling nucleic acid with said second coupling nucleic acid, wherein said second coupling nucleic acid contains a second nucleotide base sequence

140

region able to stably hybridize to a second nucleotide base sequence region of said first coupling nucleic acid under selective hybridization conditions; or

(b) one or more optional coupling nucleic acids, other than said first and second coupling nucleic acids, wherein each of said optional coupling nucleic acids is able to stably hybridize to at least two other coupling nucleic acids under selective hybridization conditions, wherein at least one of said other coupling nucleic acids may be at least one of said first and second coupling nucleic acids, and wherein each of said other coupling nucleic acids is directly or indirectly joined to said probe and said fifth nucleic acid under selective hybridization conditions,

wherein said analyte is unable to stably hybridize to any of said second nucleotide region of said probe, said first and second coupling nucleic acids, said fifth nucleic acid, and any of said optional coupling nucleic acids under said conditions,

wherein said probe is unable to stably hybridize to any of said second nucleotide region of said first coupling nucleic acid, said second coupling nucleic acid, said fifth nucleic acid, and any of said optional coupling nucleic acids under said conditions,

wherein said fifth nucleic acid is unable to stably hybridize to any of said first coupling nucleic acid, said second nucleotide region of said second coupling nucleic acid, and any of said optional coupling nucleic acids under said conditions

wherein said first coupling nucleic acid is unable to stably hybridize to said first nucleotide region of said second coupling nucleic acid under said conditions, and

wherein said second coupling nucleic acid is unable to stably hybridize to said first nucleotide region of said first coupling nucleic acid under said conditions;

b) subjecting the components of step a) to selective hybridization conditions; and

141

c) detecting said probe hybridized with said analyte as an indication of the presence or amount of said analyte in said sample.

313. The method of claim 312, wherein said probe
5 includes a label.

314. The method of claim 313, wherein said label is joined to said probe at a site located within said first nucleotide region of said probe.

315. The method of either claim 312 or 313, wherein
10 said fifth nucleic acid is immobilized by a solid support.

316. A method for detecting the presence or amount of an analyte comprising a first nucleic acid in a sample suspected of containing said sample, which comprises the steps of:

15 a) contacting the following components:

i) said sample with a probe comprising a second nucleic acid, wherein said probe contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said analyte
20 under selective hybridization conditions;

ii) said analyte with a first coupling nucleic acid comprising a third nucleic acid, wherein said first coupling nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second
25 nucleotide base sequence region of said analyte under selective hybridization conditions;

iii) a second coupling nucleic acid comprising a fourth nucleic acid with a fifth nucleic acid, wherein said fifth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a first
30 nucleotide base sequence region of said second coupling nucleic acid under selective hybridization conditions; and, alternatively,

(a) said first coupling nucleic acid with said second coupling nucleic acid, wherein said second coupling nucleic acid contains a second nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said first coupling nucleic acid under selective hybridization conditions; or

(b) one or more optional coupling nucleic acids, other than said first and second coupling nucleic acids, wherein each of said optional coupling nucleic acids is able to stably hybridize to at least two other coupling nucleic acids under selective hybridization conditions, wherein at least one of said other coupling nucleic acids may be at least one of said first and second coupling nucleic acids, and wherein each of said other coupling nucleic acids is directly or indirectly joined to said probe and said fifth nucleic acid under selective hybridization conditions,

wherein said analyte is unable to stably hybridize to any of said second nucleotide region of said first coupling nucleic acid, said second coupling nucleic acid, said fifth nucleic acid, and any of said optional coupling nucleic acids under said conditions,

wherein said probe is unable to stably hybridize to any of said second nucleotide region of said analyte, said first and second coupling nucleic acids, said fifth nucleic acid, and any of said optional coupling nucleic acids under said conditions,

wherein said fifth nucleic acid is unable to stably hybridize to any of said first coupling nucleic acid, said second nucleotide region of said second coupling nucleic acid, and any of said optional coupling nucleic acids under said conditions,

wherein said first coupling nucleic acid is unable to stably hybridize to said first nucleotide region of said second coupling nucleic acid under said conditions, and

wherein said second coupling nucleic acid is unable to stably hybridize to said first nucleotide region of said first coupling nucleic acid under said conditions;

b) subjecting the components of step a) to
5 selective hybridization conditions; and

c) detecting said probe hybridized with said analyte as an indication of the presence or amount of said analyte in said sample.

317. The method of claim 316, wherein said probe
10 further includes a label.

318. The method of claim 317, wherein said label is joined to said probe at a site located within said first nucleotide region of said probe.

319. The method of either claim 316 or 317, wherein
15 said fifth nucleic acid is immobilized by a solid support.

320. A method for detecting the presence or amount of an analyte comprising a first nucleic acid in a sample suspected of containing said sample, which comprises the steps of:

20 a) contacting the following components:

i) said sample with a probe comprising a second nucleic acid, wherein said probe contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said analyte
25 under selective hybridization conditions;

ii) said probe with a first coupling nucleic acid comprising a third nucleic acid, wherein said first coupling nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second
30 nucleotide base sequence region of said probe under selective hybridization conditions;

iii) said analyte with said first coupling nucleic acid, wherein said first coupling nucleic acid contains a

second nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under selective hybridization conditions;

iv) a second coupling nucleic acid comprising a fourth nucleic acid with a fifth nucleic acid, wherein said fifth nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said second coupling nucleic acid under selective hybridization conditions; and, alternatively,

(a) said first coupling nucleic acid with said second coupling nucleic acid, wherein said second coupling nucleic acid contains a second nucleotide base sequence region able to stably hybridize to a third nucleotide base sequence region of said first coupling nucleic acid under selective hybridization conditions; or

(b) one or more optional coupling nucleic acids, other than said first and second coupling nucleic acids, wherein each of said optional coupling nucleic acids is able to stably hybridize to at least two other coupling nucleic acids under selective hybridization conditions, wherein at least one of said other coupling nucleic acids may be at least one of said first and second coupling nucleic acids, and wherein each of said other coupling nucleic acids is directly or indirectly joined to said analyte, said probe and said fifth nucleic acid under selective hybridization conditions,

wherein said analyte is unable to stably hybridize to any of said second nucleotide region of said probe, said first and third nucleotide regions of said first coupling nucleic acid, said second coupling nucleic acid, said fifth nucleic acid, and any of said optional coupling nucleic acids under said conditions,

wherein said probe is unable to stably hybridize to any of said second nucleotide region of said analyte, said second and third nucleotide regions of said first coupling nucleic acid, said second coupling nucleic acid, said

145

fifth nucleic acid, and any of said optional coupling nucleic acids under said conditions,

wherein said fifth nucleic acid is unable to stably hybridize to any of said first coupling nucleic acid, said
5 second nucleotide region of said second coupling nucleic acid, and any of said optional coupling nucleic acids under said conditions,

wherein said first coupling nucleic acid is unable to stably hybridize to said second nucleotide region of said
10 second coupling nucleic acid, and

wherein said second coupling nucleic acid is unable to stably hybridize to either said first or second nucleotide region of said first coupling nucleic acid;

b) subjecting the components of step a) to
15 selective hybridization conditions; and

c) detecting said probe hybridized with said analyte as an indication of the presence or amount of said analyte in said sample.

321. The method of claim 320, wherein said probe
20 further includes a label.

322. The method of claim 321, wherein said label is joined to said probe at a site located within said first nucleotide region of said probe.

323. The method of either claim 320 or 321, wherein
25 said fifth nucleic acid is immobilized by a solid support.

324. A method of amplifying a target comprising a first nucleic acid, which comprises the steps of:

a) contacting a sample suspected of containing said target with a second nucleic acid, wherein said second
30 nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a first nucleotide base sequence region of said target under amplification conditions, and wherein said first nucleotide region of said

second nucleic acid includes one or more modified nucleotides;

b) subjecting the components of step a) to said amplification conditions, so that

5 i) the hybridization binding affinity between said target and said second nucleic acid is greater than the hybridization binding affinity between said target and an unmodified form of said second nucleic acid, and

10 ii) the hybridization rate between said target and said second nucleic acid is greater than the hybridization rate between said target and an unmodified form of said second nucleic acid; and

c) incubating the components of step a) under said amplification conditions, such that said target is
15 amplified.

325. The method of claim 324, wherein said first nucleotide region of said second nucleic acid includes one or more clusters of at least about 4 modified nucleotides.

20 326. The method of claim 324, wherein said first nucleotide region of said second nucleic acid includes one or more clusters of at least about 6 modified nucleotides.

327. The method of claim 324, wherein said first nucleotide region of said second nucleic acid includes one or more clusters of at least about 8 modified nucleotides.

25 328. The method of claim 324, wherein substantially all of the nucleotides contained in said first nucleotide region of said second nucleic acid are modified.

329. The method of claim 325, wherein at least one of said modified nucleotides includes a modification selected
30 from the group consisting of:

- a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;

147

- c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage;
- and
- e) a modification to the internucleotide linkage.

5 330. The method of claim 325, wherein at least one of said modified nucleotides includes two modifications selected from the group consisting of:

- a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;
- 10 c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage;
- and
- e) a modification to the internucleotide linkage.

15 331. The method of claim 325, wherein at least one of said modified nucleotides includes a 2'-modification to the ribofuranosyl moiety selected from the group consisting of:

- a) an alkyl substitution;
- b) an alkoxy substitution; and
- 20 c) a halide substitution.

332. The method of claim 325, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

25 333. The method of claim 325, wherein at least one of said modified nucleotides includes a propyne substitution to the nitrogenous base.

334. The method of claim 333, wherein said propyne substitution is to a cytidine analog.

30 335. The method of claim 333, wherein said propyne substitution is to a thymidine analog.

336. The method of claim 325, wherein each said modified nucleotide of one or more of said clusters of said first nucleotide region of said second nucleic acid contains the same modification.

5 337. The method of claim 336, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

338. The method of claim 324, wherein said second nucleic acid includes one or more conjugate molecules.

10 339. The method of claim 325, wherein said second nucleic acid includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to said second nucleic acid at a site located within one or more of said clusters contained in said first nucleotide region of said second nucleic acid.
15

340. The method of claim 325, wherein said contacting step further includes contacting said second nucleic acid with nucleotide triphosphates and at least one nucleic acid polymerase.

20 341. The method of claim 340, wherein said second nucleic acid is capable of having nucleotide bases added to its 3' terminus by at least one of said polymerases.

342. The method of claim 325 or 341, wherein said contacting step further includes contacting said target
25 with a third nucleic acid, wherein said third nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said target under selective hybridization conditions,

30 wherein said second nucleic acid is unable to stably hybridize to either said second nucleotide region of said

target or said third nucleic acid under said hybridization conditions, and

wherein said third nucleic acid is unable to stably hybridize to said first nucleotide region of said target
5 under said conditions.

343. The method of claim 342, wherein the formation of a stable hybrid between said third nucleic acid and said target under said conditions reduces or prevents amplification of said target.

10 344. The method of claim 342, wherein said first and second nucleotide regions of said target constitute substantially the same nucleotide region.

345. The method of claim 342, wherein said first nucleotide region of said third nucleic acid includes one
15 or more modified nucleotides.

346. The method of claim 342, wherein said first nucleotide region of said third nucleic acid includes one or more clusters of at least about 4 modified nucleotides.

347. The method of claim 342, wherein substantially
20 all of the nucleotides contained in said first nucleotide region of said third nucleic acid are modified.

348. The method of claim 347, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

25 349. The method of claim 347, wherein each said modified nucleotide of one or more of said clusters contains the same modification.

150

350. The method of claim 349, wherein said modification consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

351. The method of claim 342, wherein at least one of
5 said second and third nucleic acids includes one or more conjugate molecules.

352. The method of claim 346, wherein at least one of
said second and third nucleic acids includes one or more
conjugate molecules, and wherein at least one of said con-
10 jugate molecules is joined to at least one of said second
and third nucleic acids at a site located within one or
more of said clusters contained in at least one of said
first nucleotide region of said second nucleic acid and
said first nucleotide region of said third nucleic acid.

15 353. The method of claim 342, wherein said second
nucleic acid contains a 5' nucleotide base sequence region
which is unable to stably hybridize to said target under
said conditions.

354. The method of claim 353, wherein any of said
20 target and said second and third nucleic acids is directly
or indirectly immobilized by a solid support.

355. A kit comprising a nucleic acid probe for use in
detecting the presence or amount of a nucleic acid analyte
in a sample suspected of containing said analyte, wherein
25 said probe comprises a first nucleotide base sequence
region able to stably hybridize to a first nucleotide base
sequence region of said analyte under selective hybridiza-
tion conditions, and wherein said first nucleotide region
of said probe includes at least about 4 modified nucleo-
30 tide bases, so that

a) the hybridization binding affinity between said
analyte and said probe is greater than the hybridization

151

binding affinity between said analyte and an unmodified form of said probe, under said conditions, and

- b) the hybridization rate between said analyte and said probe is greater than the hybridization rate between
5 said analyte and an unmodified form of said probe, under said conditions.

356. The kit of claim 355, wherein said first nucleotide region of said probe includes one or more clusters of at least about 6 modified nucleotides.

- 10 357. The kit of claim 355, wherein said first nucleotide region of said probe includes one or more clusters of at least about 8 modified nucleotides.

358. The kit of claim 355, wherein substantially all of the nucleotides contained in said first nucleotide
15 region of said probe are modified.

359. The kit of claim 355, wherein at least one of said modified nucleotide bases contains a modification selected from the group consisting of:

- a) a modification to the nitrogenous base;
20 b) a modification to the sugar moiety;
c) a modification to phosphate moiety;
d) a modification to the internucleoside linkage;
and
e) a modification to the internucleotide linkage
25 moiety.

360. The kit of claim 355, wherein at least one of said modified nucleotides includes two modifications selected from the group consisting of:

- a) a modification to the nitrogenous base;
30 b) a modification to the sugar moiety;
c) a modification to phosphate moiety;

d) a modification to the internucleoside linkage;
and

e) a modification to the internucleotide linkage moiety.

5 361. The kit of claim 355, wherein at least one of said modified nucleotides contains a 2'-modification to the ribofuranosyl moiety selected from the group consisting of:

- a) an alkyl substitution;
- 10 b) an alkoxy substitution; and
- c) a halide substitution.

362. The kit of claim 355, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

15 363. The kit of claim 355, wherein at least one of said modified nucleotides includes a propyne substitution to the nitrogenous base.

364. The kit of claim 363, wherein said propyne substitution is to a cytidine analog.

20 365. The kit of claim 363, wherein said propyne substitution is to a thymidine analog.

366. The kit of claim 355, wherein said probe includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to said
25 probe at a site located within one or more of said clusters contained in said first nucleotide region of said probe.

367. The kit of claim 355, wherein said probe consists of from about 10 to about 100 nucleotides.

153

368. The kit of claim 355, wherein said probe consists of from about 10 to about 15 nucleotides.

369. The kit of claim 355, wherein said probe consists of from about 12 to about 15 nucleotides.

5 370. The kit of claim 355, wherein said probe further includes a label.

371. The kit of claim 370, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- 10 b) an enzyme,
- c) an enzyme cofactor,
- d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- 15 g) a chemiluminescent molecule,
- h) a fluorescent chemiluminescent molecule,
- i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- 20 l) a base sequence region that is unable to stably hybridize to said analyte under said conditions.

372. The kit of claim 371, wherein said label is a chemiluminescent molecule.

373. The kit of claim 372, wherein light is emitted
25 by said chemiluminescent molecule upon the return to ground state of an electronically excited N-acridone.

374. The kit of claim 372, wherein said label is an acridinium ester derivative.

375. The kit of either claim 355 or 370, further
30 comprising a solid support.

376. The kit of claim 375, wherein said probe is directly or indirectly immobilized by said solid support.

377. The kit of claim 355 or 370, further comprising one or more nucleic acid controls.

5 378. The kit of claim 355 or 370, further comprising one or more hybridization reagents.

379. A kit comprising a first nucleic acid capable of amplifying an analyte comprising a second nucleic acid in a sample suspected of containing said analyte, wherein
10 said first nucleic acid contains a first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte under amplification conditions, and wherein said first nucleotide region of said first nucleic acid includes at least
15 about 4 modified nucleotides, so that

a) the hybridization binding affinity between said analyte and said first nucleic acid is greater than the hybridization binding affinity between said analyte and an unmodified form of said first nucleic acid, under said
20 conditions, and

b) the hybridization rate between said analyte and said first nucleic acid is greater than the hybridization rate between said analyte and an unmodified form of said first nucleic acid, under said conditions.

25 380. The kit of claim 379, wherein said first nucleotide region of said first nucleic acid includes one or more clusters of at least about 6 modified nucleotides.

381. The kit of claim 379, wherein said first nucleotide region of said first nucleic acid includes one or
30 more clusters of at least about 8 modified nucleotides.

155

382. The kit of claim 379, wherein substantially all of the nucleotides contained in said first nucleotide region of said first nucleic acid are modified.

383. The kit of claim 379, wherein at least one of said modified nucleotide bases contains a modification selected from the group consisting of:

- a) a modification to the nitrogenous base;
 - b) a modification to the sugar moiety;
 - c) a modification to phosphate moiety;
 - 10 d) a modification to the internucleoside linkage;
- and
- e) a modification to the internucleotide linkage moiety.

384. The kit of claim 379, wherein at least one of said modified nucleotides includes two modifications selected from the group consisting of:

- a) a modification to the nitrogenous base;
 - b) a modification to the sugar moiety;
 - c) a modification to phosphate moiety;
 - 20 d) a modification to the internucleoside linkage;
- and
- e) a modification to the internucleotide linkage moiety.

385. The kit of claim 379, wherein at least one of said modified nucleotides contains a 2'-modification to the ribofuranosyl moiety selected from the group consisting of:

- a) an alkyl substitution;
- b) an alkoxy substitution; and
- 30 c) a halide substitution.

386. The kit of claim 379, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

156

387. The kit of claim 379, wherein at least one of said modified nucleotides includes a propyne substitution to the nitrogenous base.

388. The kit of claim 387, wherein said propyne
5 substitution is to a cytidine analog.

389. The kit of claim 387, wherein said propyne substitution is to a thymidine analog.

390. The kit of claim 379, wherein said first nucleic
acid includes one or more conjugate molecules, and wherein
10 at least one of said conjugate molecules is joined to said
first nucleic acid at a site located within one or more of
said clusters contained in said first nucleotide region of
said first nucleic acid.

391. The kit of claim 379, further comprising a solid
15 support.

392. The kit of claim 391, wherein said first nucleic
acid is directly or indirectly immobilized by said solid
support.

393. The kit of claim 392, further comprising nucle-
20 tide triphosphates and at least one nucleic acid
polymerase.

394. The kit of claim 393, wherein said first nucleic
acid is capable of having nucleotide bases added to its 3'
terminus by at least one of said nucleic acid polymerases.

25 395. The kit of claim 379, further comprising one or
more amplification reagents.

396. The kit of claim 379, further comprising a third
nucleic acid, wherein said third nucleic acid contains a

first nucleotide base sequence region able to stably hybridize to a second nucleotide base sequence region of said analyte, such that said analyte is able to stably hybridize to said third nucleic acid under selective
5 hybridization conditions,

wherein said first oligonucleotide is unable to stably hybridize to either said second nucleotide region of said analyte or said third nucleic acid under said hybridization conditions, and

10 wherein said third nucleic acid is unable to stably hybridize to said first nucleotide region of said analyte under said conditions.

397. The kit of claim 396, wherein the formation of a stable hybrid between said third nucleic acid and said
15 analyte under said conditions reduces or prevents amplification of said analyte.

398. The kit of claim 396, wherein said first and second nucleotide regions of said analyte constitute substantially the same nucleotide region.

20 399. The kit of claim 396, further comprising one or more hybridization reagents.

400. A nucleic acid probe comprising a nucleotide base sequence region containing one or more clusters of at least about 4 modified nucleotides, wherein said probe
25 includes at least one label joined to said probe at a site located within one of said clusters contained in said nucleotide region.

401. The probe of claim 400, wherein said nucleotide region of said probe includes one or more clusters of at
30 least about 6 modified nucleotides.

402. The probe of claim 400, wherein said nucleotide region of said probe includes one or more clusters of at least about 8 modified nucleotides.

403. The probe of claim 400, wherein substantially
5 all of the nucleotides contained in said nucleotide region of said probe are modified.

404. The probe of claim 400, wherein at least one of said modified nucleotides includes a modification selected from the group consisting of:

- 10 a) a modification to the nitrogenous base;
- b) a modification to the sugar moiety;
- c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage;
- and
- 15 e) a modification to the internucleotide linkage.

405. The probe of claim 400, wherein at least one of said modified nucleotides includes two modifications selected from the group consisting of:

- a) a modification to the nitrogenous base;
- 20 b) a modification to the sugar moiety;
- c) a modification to the phosphate moiety;
- d) a modification to the internucleoside linkage;
- and
- e) a modification to the internucleotide linkage.

25 406. The probe of claim 400, wherein at least one of said modified nucleotides includes a 2'-modification to the ribofuranosyl moiety selected from the group consisting of:

- a) an alkyl substitution;
- 30 b) an alkoxy substitution; and
- c) a halide substitution.

407. The probe of claim 400, wherein at least one of said modified nucleotides includes a 2'-O-methyl substitution to the ribofuranosyl moiety.

408. The probe of claim 400, wherein at least one of
5 said modified nucleotides includes a propyne substitution to the nitrogenous base.

409. The probe of claim 408, wherein said propyne substitution is to a cytidine analog.

410. The probe of claim 408, wherein said propyne
10 substitution is to a thymidine analog.

411. The probe of claim 400, wherein each said modified nucleotide of one or more of said clusters contains the same modification.

412. The probe of claim 411, wherein said modifica-
15 tion consists of a 2'-O-methyl substitution to the ribofuranosyl moiety.

413. The probe of claim 400, wherein said probe includes one or more conjugate molecules, and wherein at least one of said conjugate molecules is joined to said
20 probe at a site located within one or more of said clusters contained in said nucleotide region of said probe.

414. The probe of claim 400, wherein said probe is an oligonucleotide consisting of from about 10 to about 100 nucleotide bases.

25 415. The probe of claim 400, wherein said probe is an oligonucleotide consisting of from about 10 to about 15 nucleotide bases.

160

416. The probe of claim 400, wherein said probe is an oligonucleotide consisting of from about 12 to about 15 nucleotide bases.

417. The probe of claim 400, wherein said probe
5 further includes a label.

418. The probe of claim 417, wherein said label is selected from the group consisting of:

- a) a radioisotope,
- b) an enzyme,
- 10 c) an enzyme cofactor,
- d) an enzyme substrate,
- e) a dye,
- f) a hapten,
- g) a chemiluminescent molecule,
- 15 h) a fluorescent molecule,
- i) a phosphorescent molecule,
- j) an electrochemiluminescent molecule,
- k) a chromophore, and
- l) a nucleotide base sequence region that is unable
20 to stably hybridize to said analyte under said conditions.

419. The probe of claim 418, wherein said label is a chemiluminescent molecule.

420. The probe of claim 419, wherein light is emitted by said chemiluminescent molecule upon the return to
25 ground state of an electronically excited N-acridone.

421. The probe of claim 419, wherein said chemiluminescent molecule is an acridinium ester derivative.

1/9

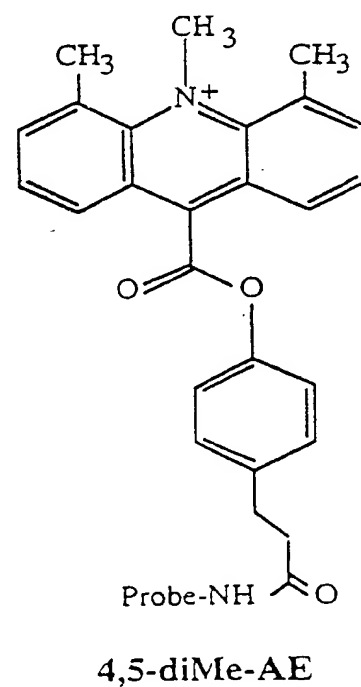
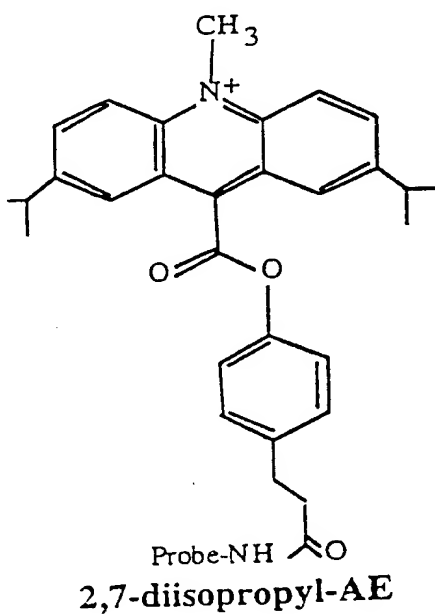
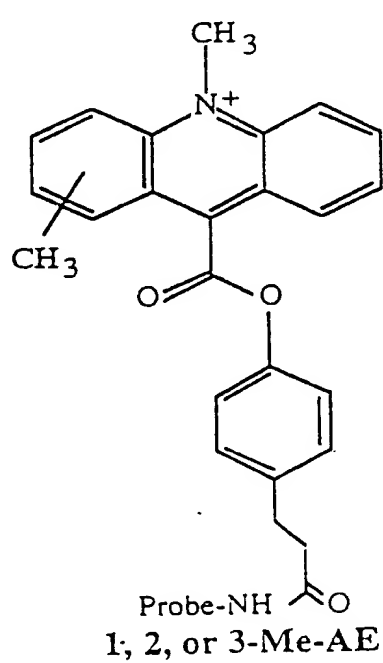
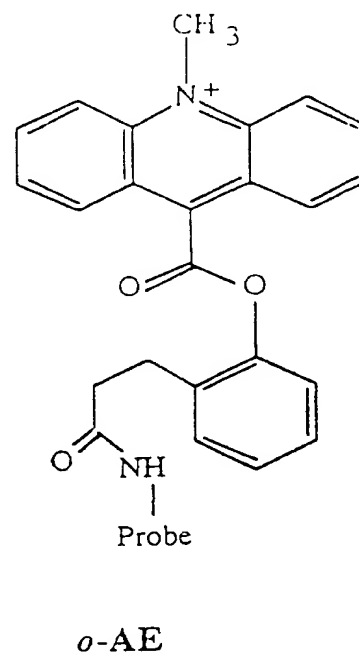
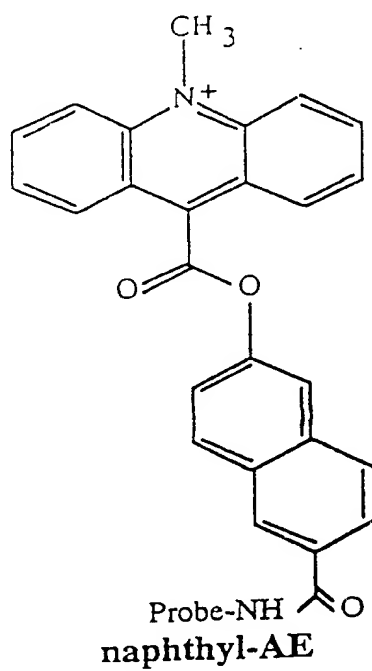
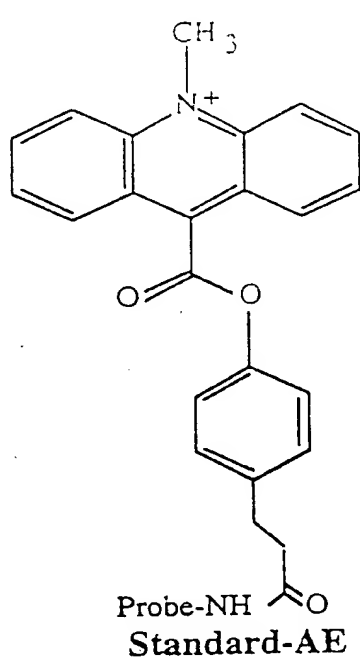
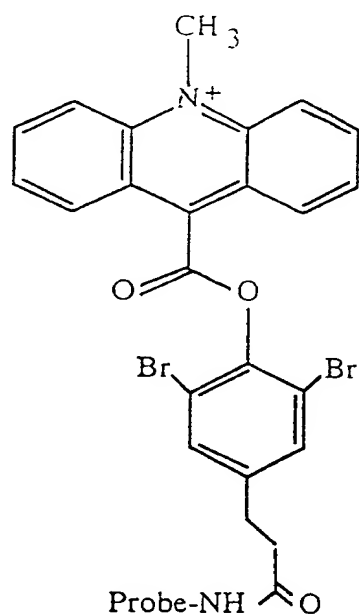
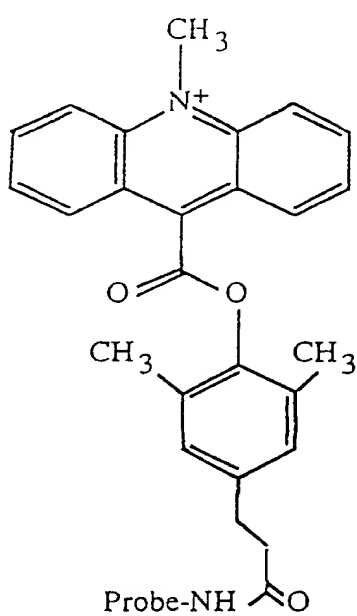
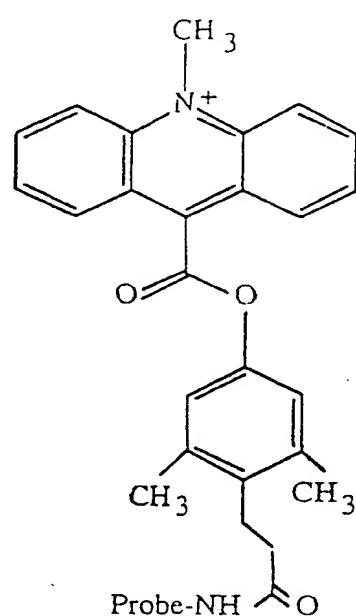
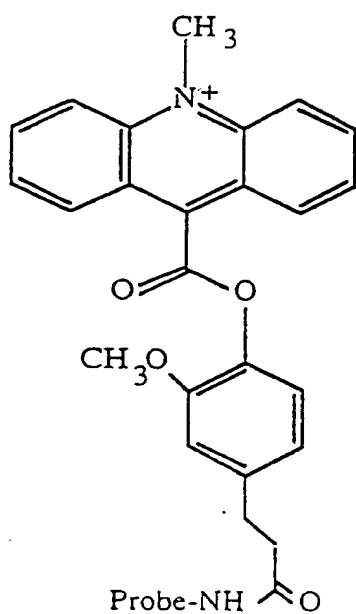
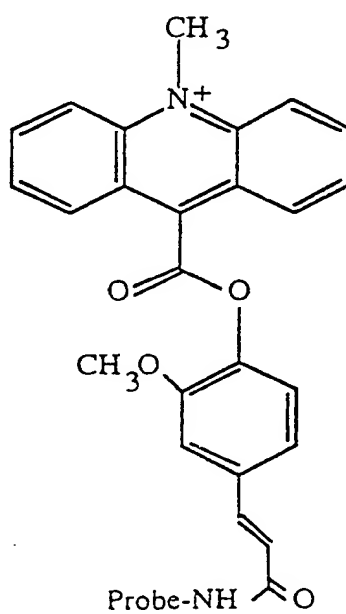
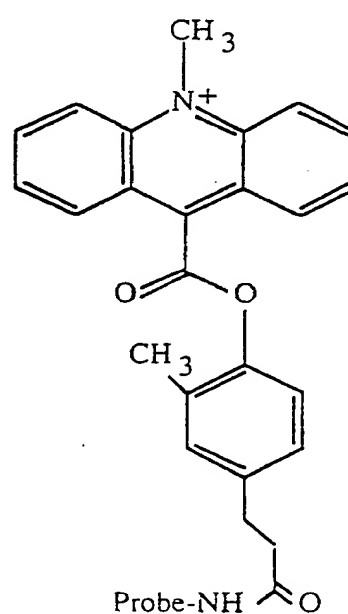
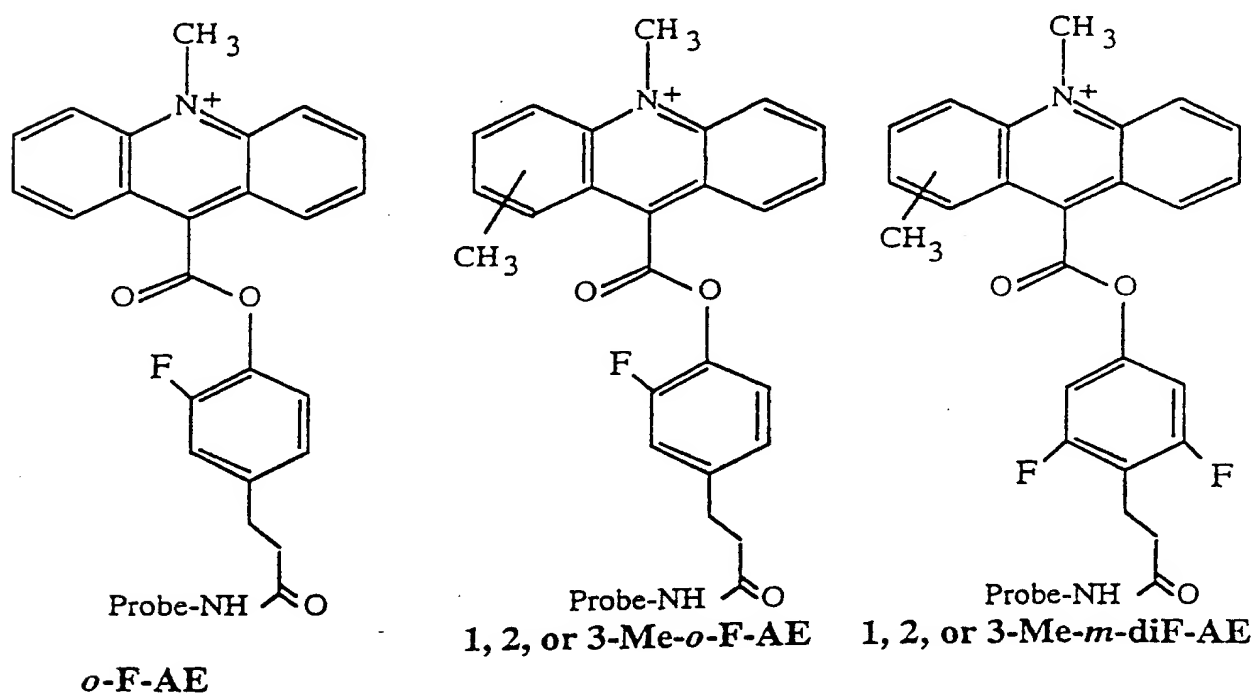


Fig. 1A

2/9

*o*-diBr-AE*o*-diMe-AE*m*-diMe-AE*o*-MeO-AE*o*-MeO (cinnamyl)-AE*o*-Me-AE**Fig. 1B**

**Fig. 1C**

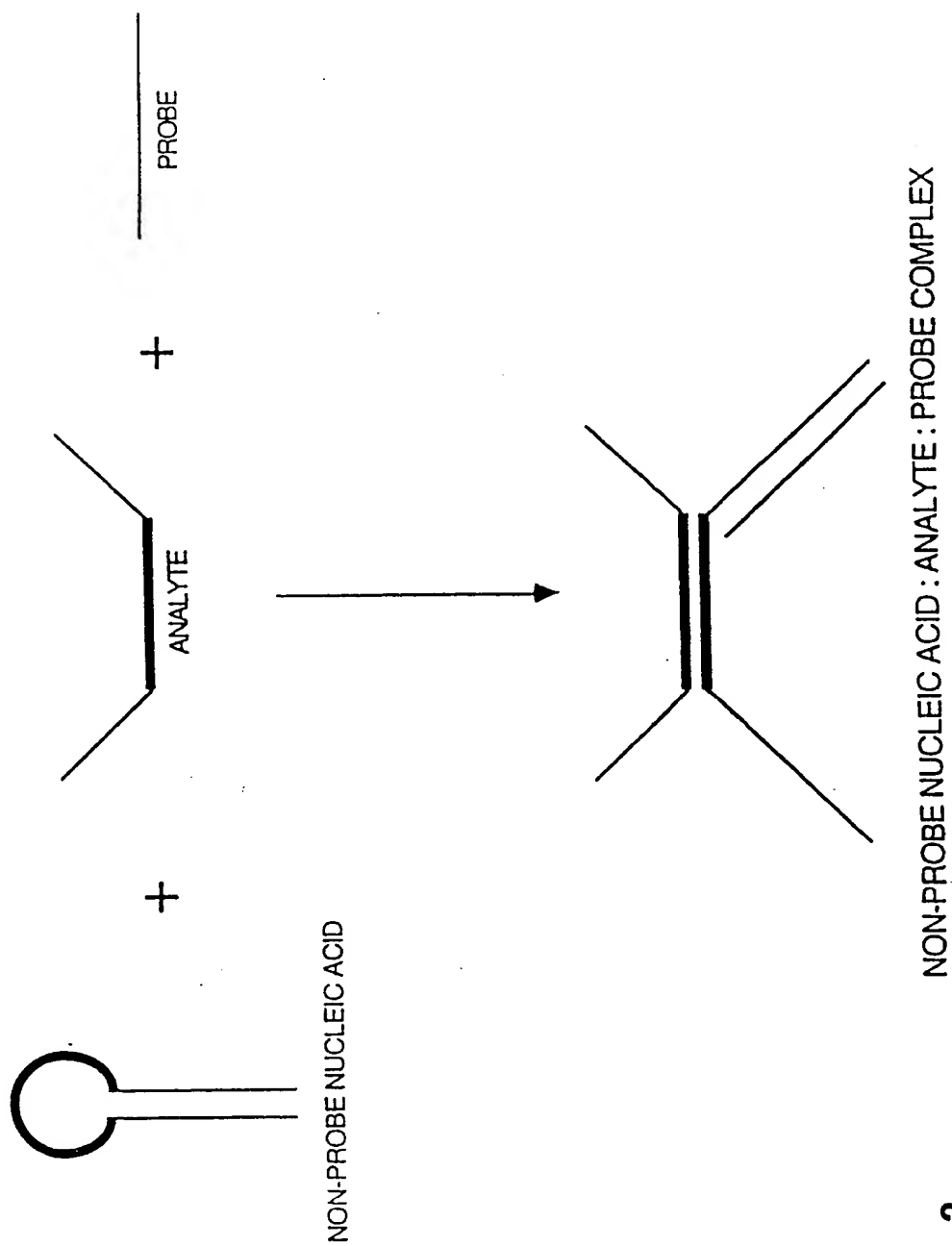
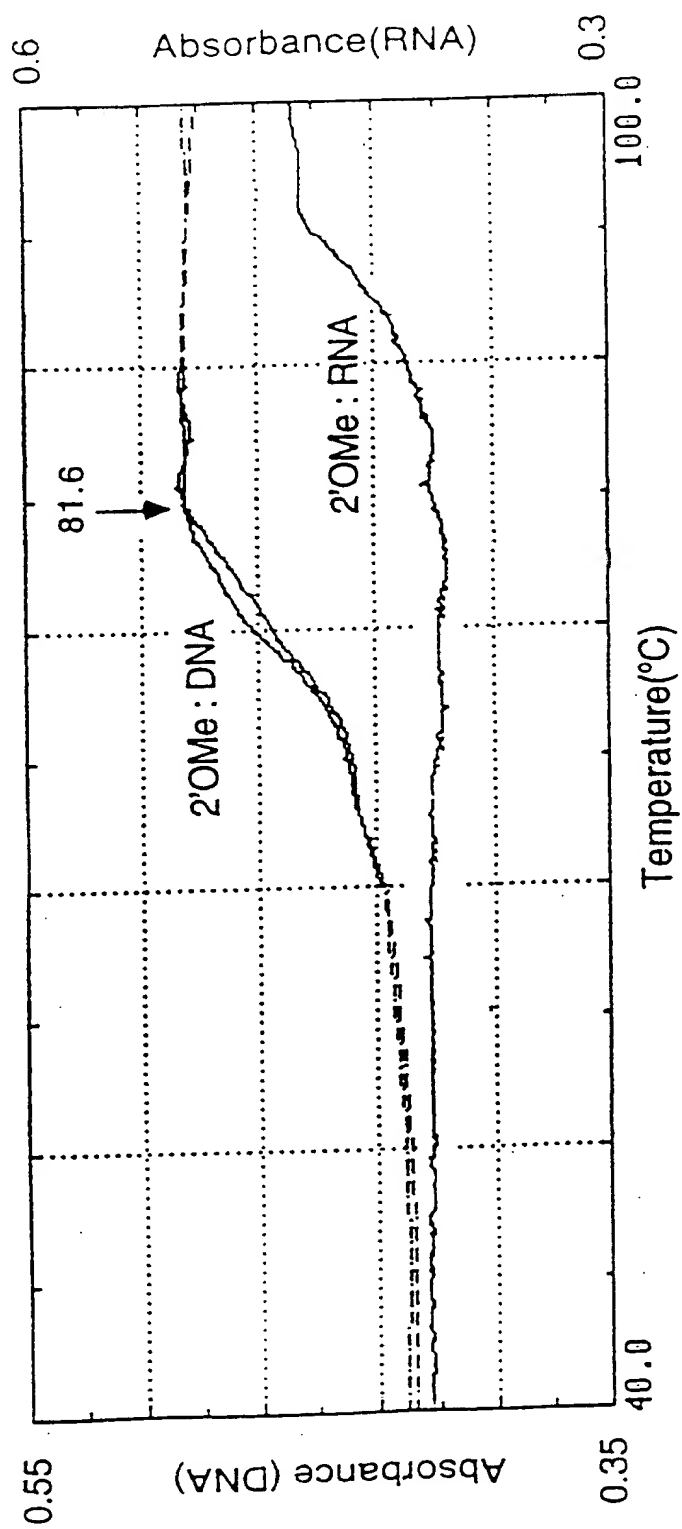
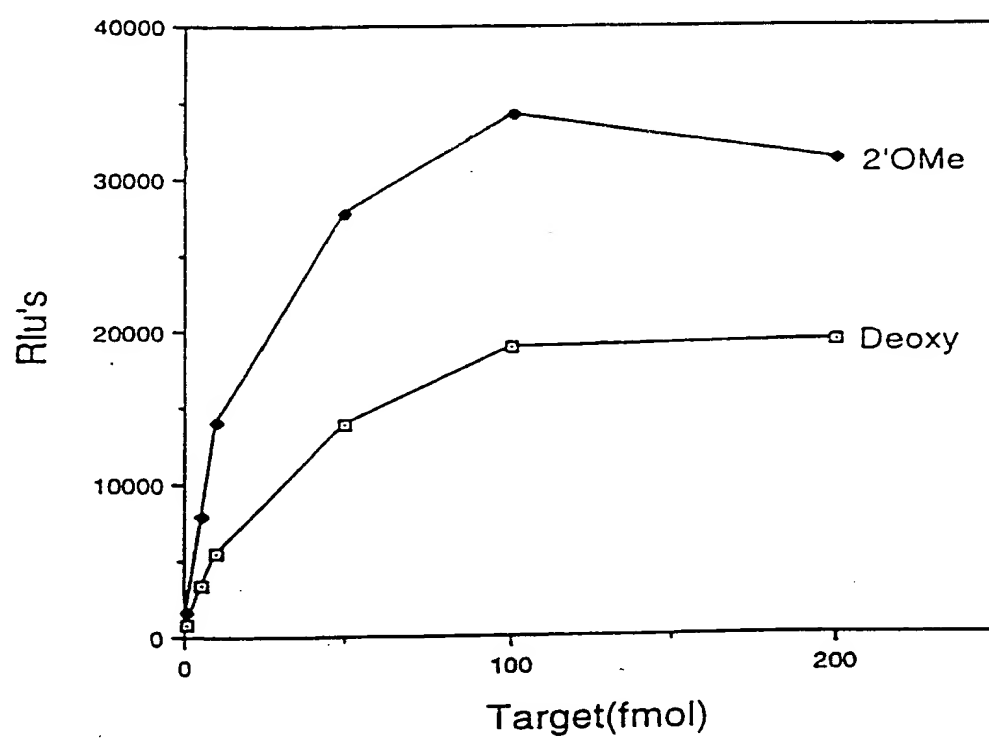


Fig. 2

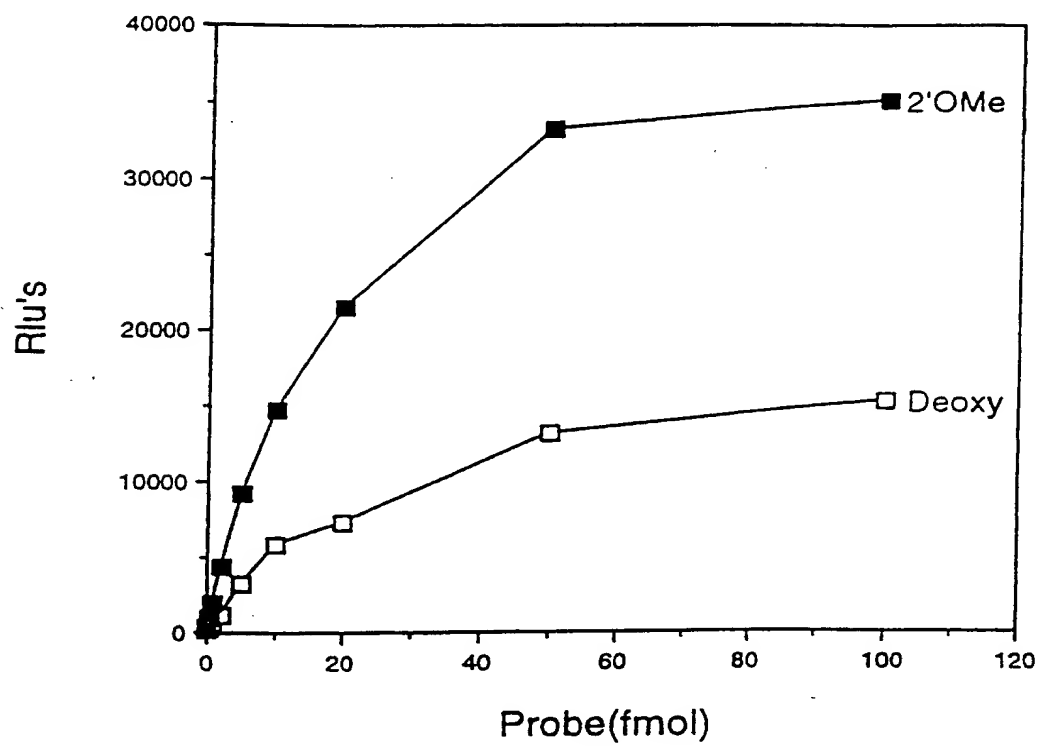
5/9

**Fig. 3**

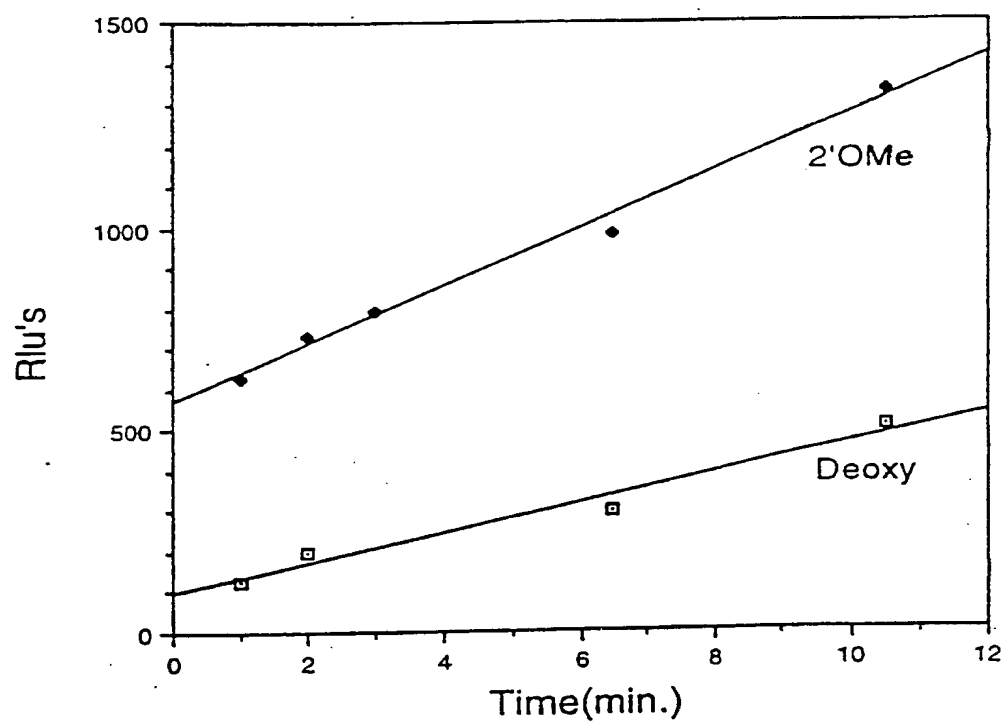
6/9

**Fig. 4**

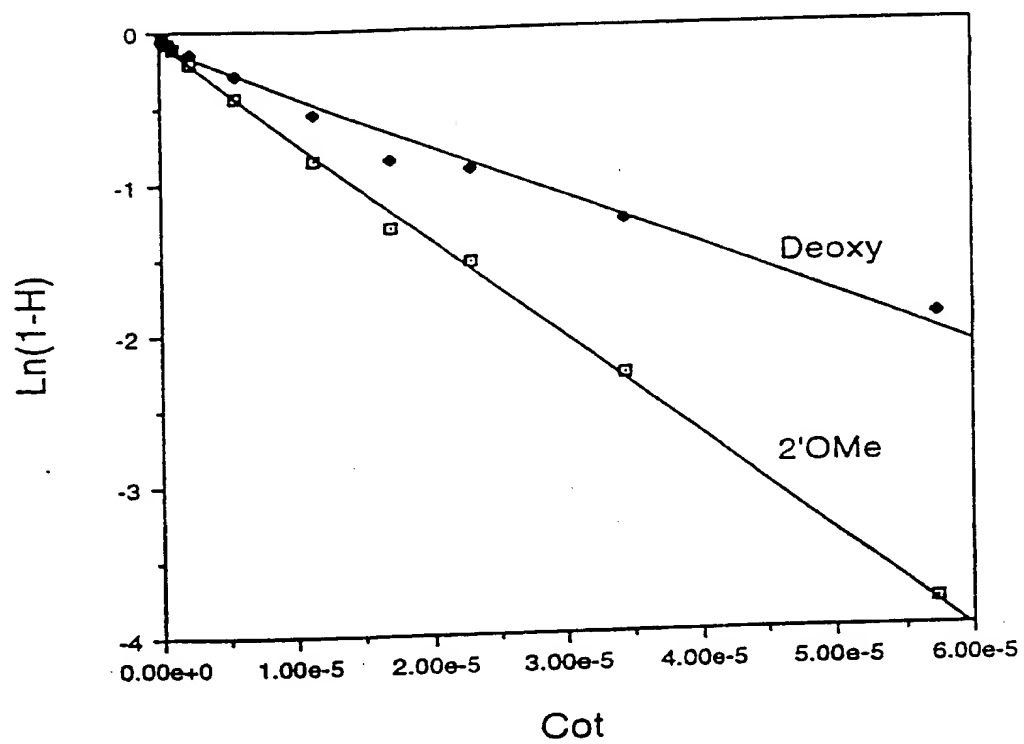
7/9

**Fig. 5**

8/9

**Fig. 6**

9/9

*Fig. 7*

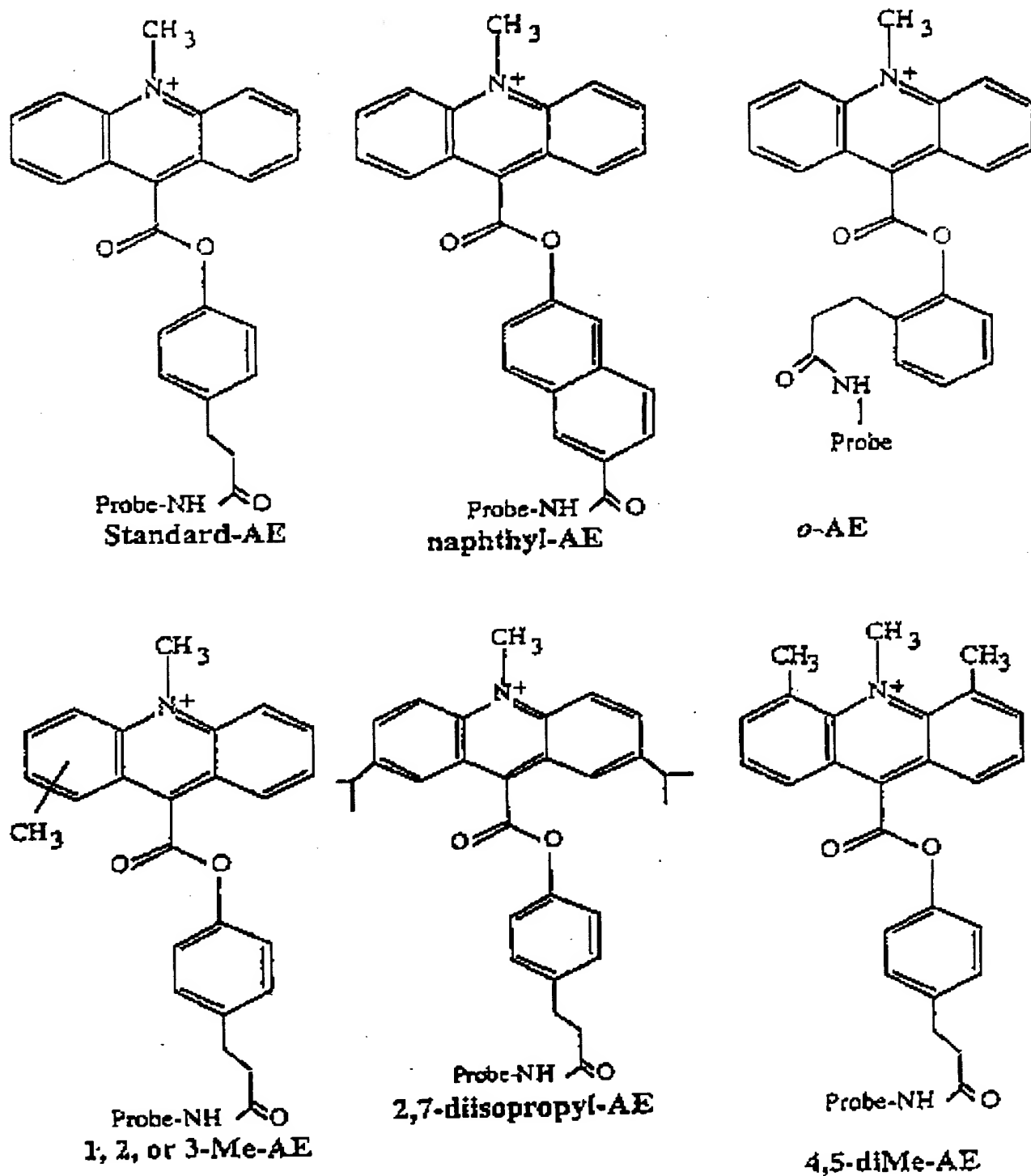


FIGURE 1A

2/9

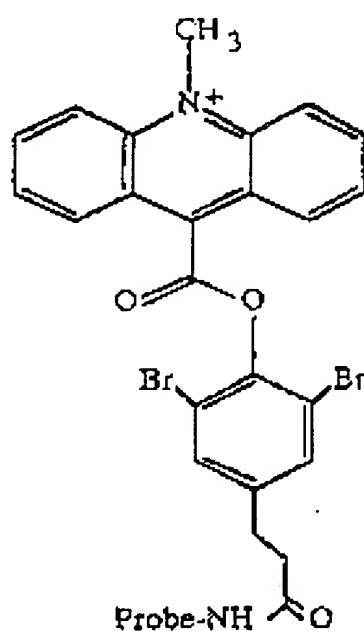
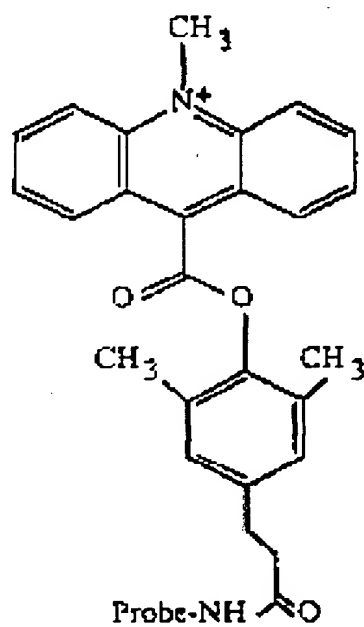
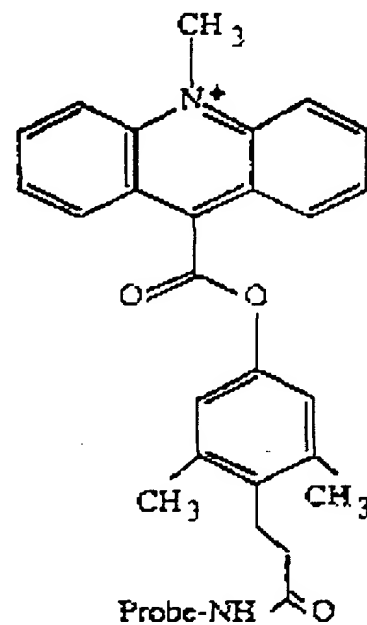
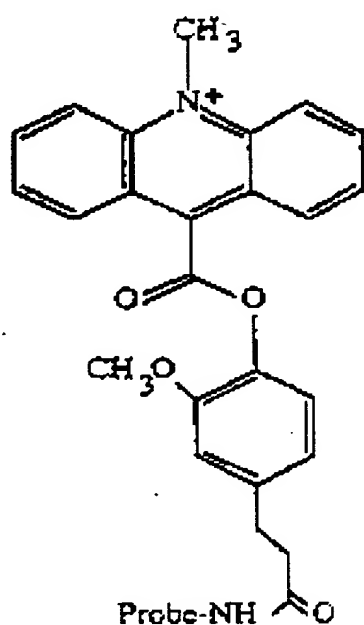
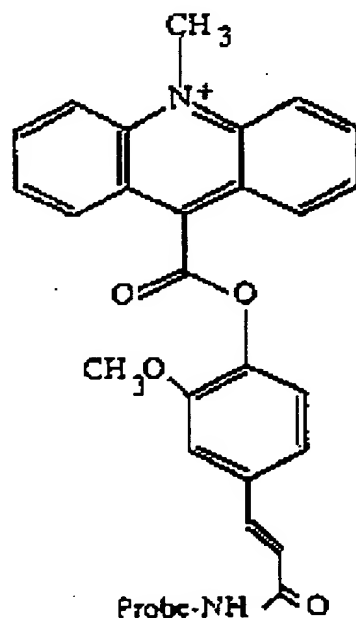
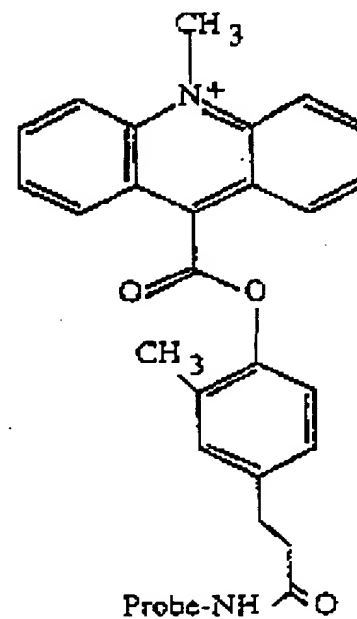
*o*-diBr-AE*o*-diMe-AE*m*-diMe-AE*o*-MeO-AE*o*-MeO (cinnamyl)-AE*o*-Me-AE

FIGURE 1B

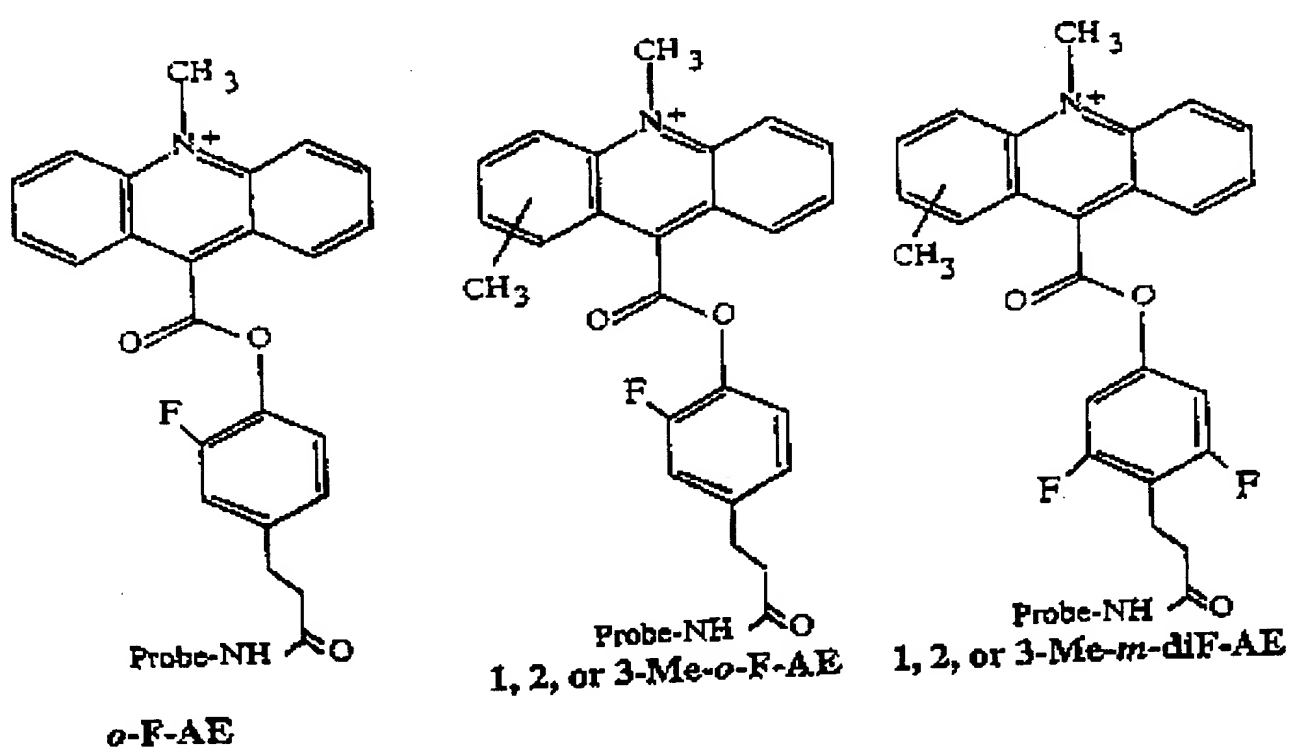


FIGURE 1C

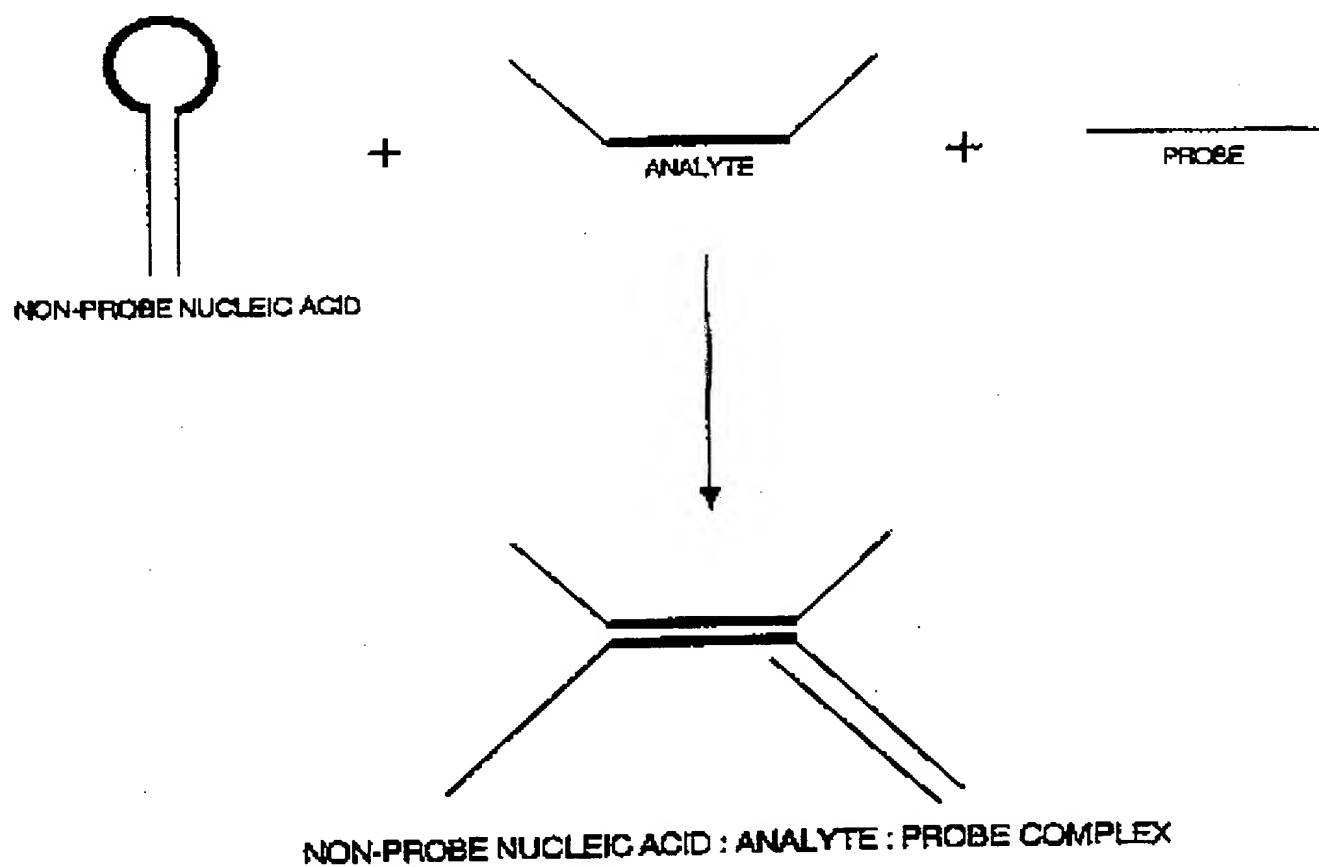


FIGURE 2

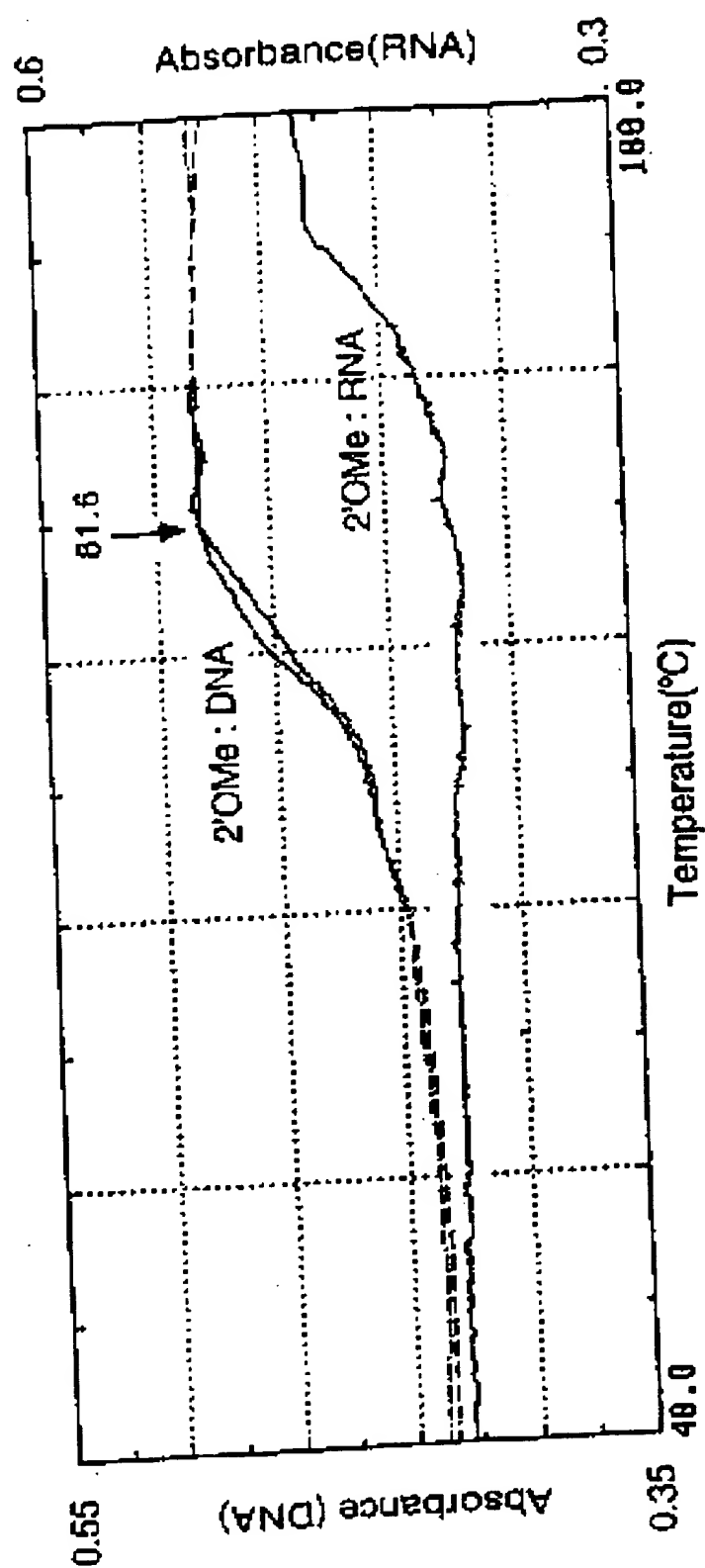


FIGURE 3

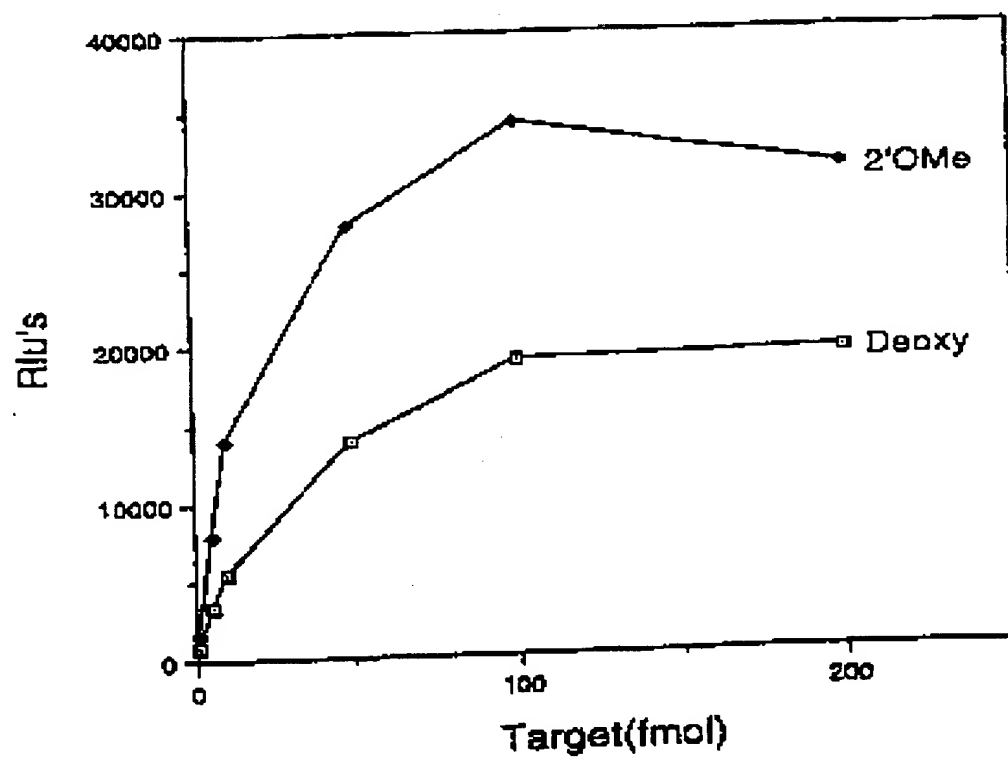


FIGURE 4

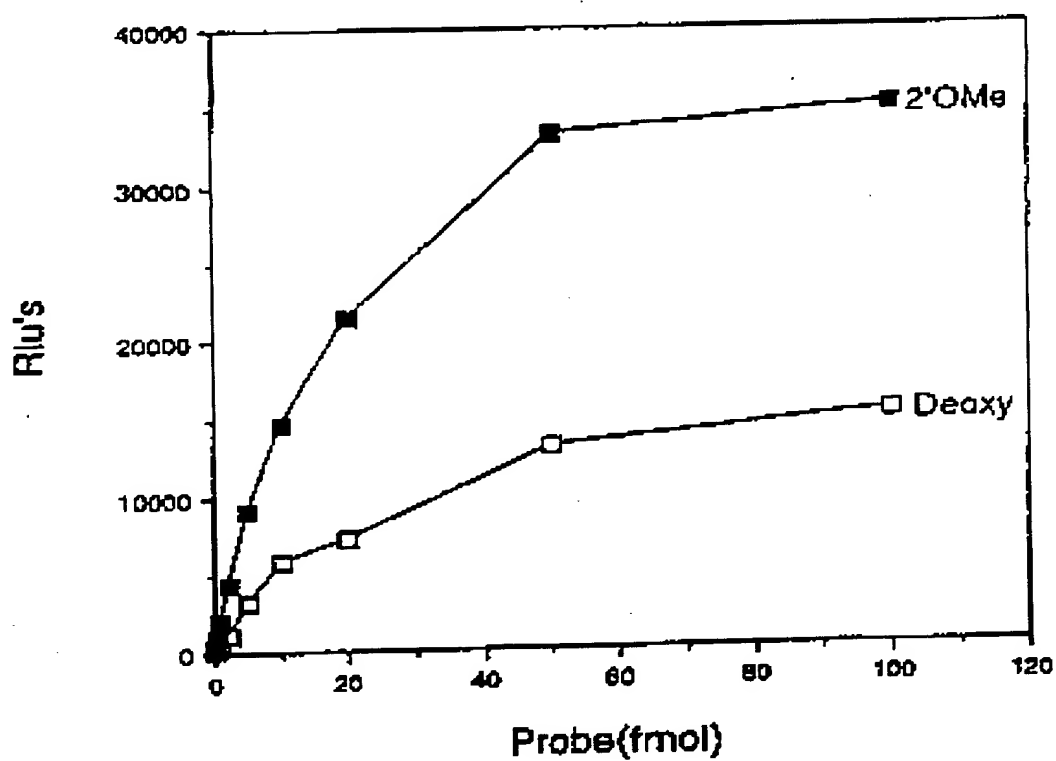


FIGURE 5

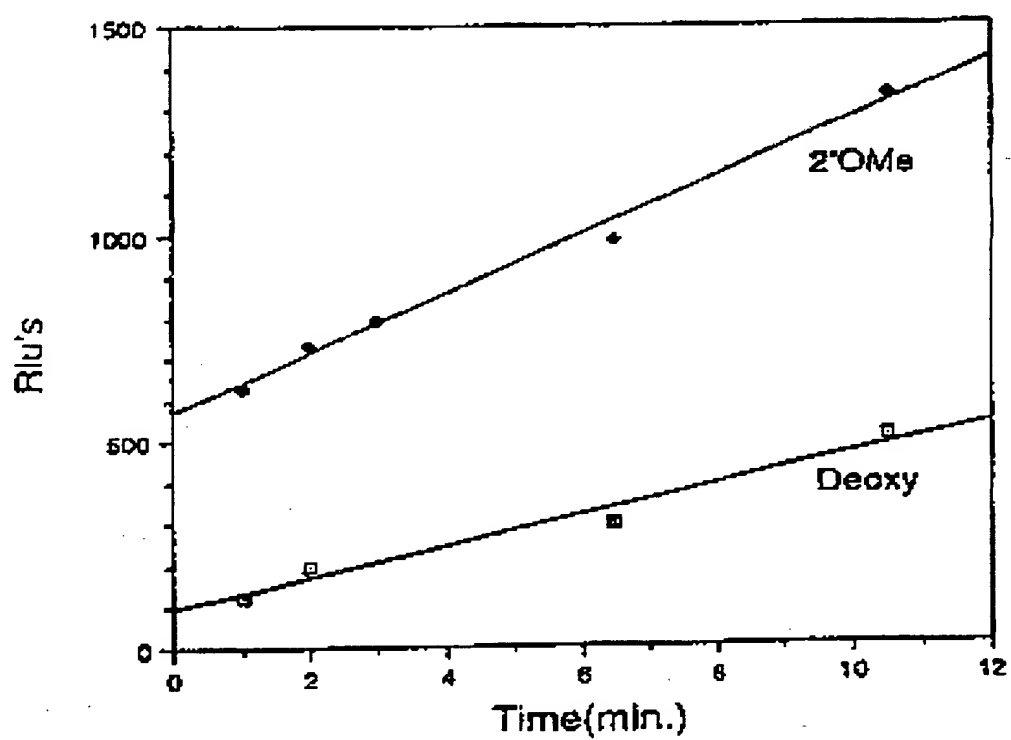


FIGURE 6

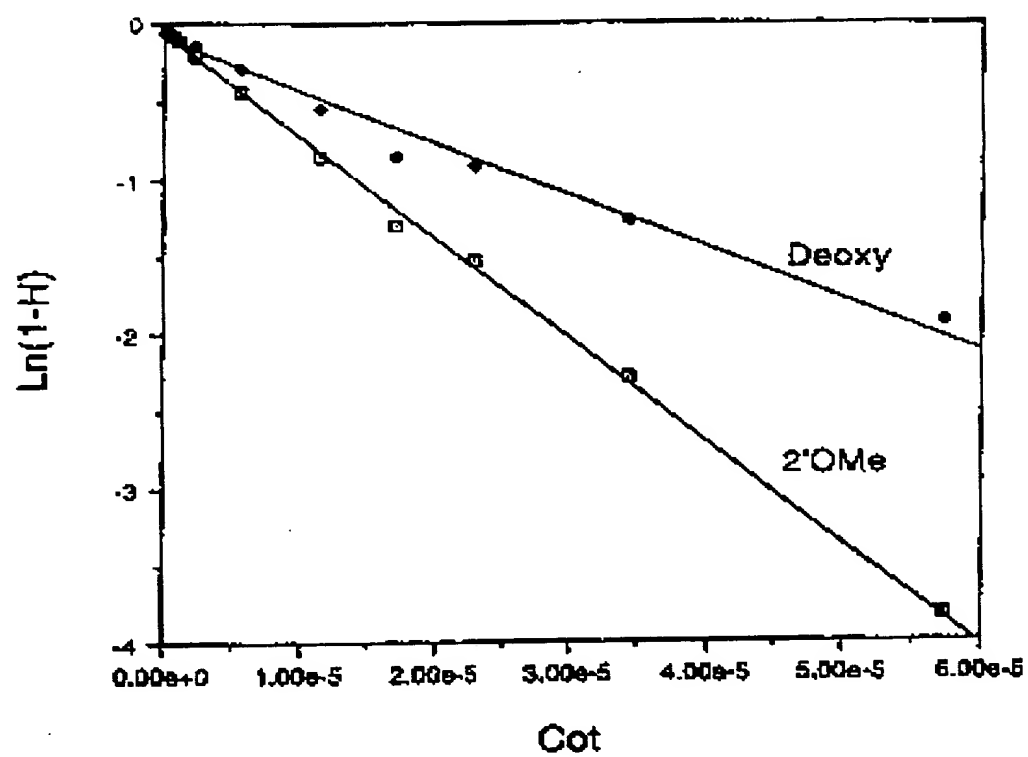


FIGURE 7

**CORRECTED
VERSION***

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/68, C07H 21/00	A3	(11) International Publication Number: WO 98/02582 (43) International Publication Date: 22 January 1998 (22.01.98)
(21) International Application Number: PCT/US97/12347 (22) International Filing Date: 15 July 1997 (15.07.97) (30) Priority Data: 60/021,818 16 July 1996 (16.07.96) US (71) Applicant: GEN-PROBE INCORPORATED [US/US]; 10210 Genetic Center Drive, San Diego, CA 92121 (US). (72) Inventors: BECKER, Michael, McClellan; 12819 Meadowdale Lane, San Diego, CA 92131 (US). MAJLESSI, Mehrdad; 15895 Sunnyfield Place, San Diego, CA 92127 (US). (74) Agents: HEBER, Sheldon, O. et al.; Lyon & Lyon LLP, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).		(81) Designated States: AU, CA, JP, KR, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> (88) Date of publication of the international search report: 2 July 1998 (02.07.98)
(54) Title: METHODS FOR DETECTING AND AMPLIFYING NUCLEIC ACID SEQUENCES USING MODIFIED OLIGONUCLEOTIDES HAVING INCREASED TARGET SPECIFIC T _m		
(57) Abstract The present invention concerns oligonucleotides containing one or more modified nucleotides which increase the binding affinity of the oligonucleotides to target nucleic acids having a complementary nucleotide base sequence. These modified oligonucleotides hybridize to the target sequence at a faster rate than unmodified oligonucleotides having an identical nucleotide base sequence. Such modified oligonucleotides include oligonucleotides containing at least one 2'-O-methylribofuranosyl moiety joined to a nitrogenous base. Oligonucleotides can be modified in accordance with the present invention to preferentially bind RNA targets. The present invention also concerns methods of using these modified oligonucleotides and kits containing the same.		

*(Referred to in PCT Gazette No. 21/1998, Section II)

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/12347

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C07H21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 32305 A (DAKO AS) 30 November 1995 see page 2, line 34 - page 6, line 32 ---	1-299, 324-421
A	WO 95 14706 A (ISIS PHARMACEUTICALS INC) 1 June 1995 see page 12, line 13 - page 13, line 7 ---	1-299, 324-421
A	EP 0 415 901 A (MONSANTO CO) 6 March 1991 see page 11, line 56 - page 12, line 38 ---	1-299, 324-421
A	WO 93 13121 A (ISIS PHARMACEUTICALS INC) 8 July 1993 cited in the application see page 2, line 23 - page 5, line 8 --- -/--	1-299, 324-421

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

Z document member of the same patent family

3

Date of the actual completion of the international search

9 April 1998

Date of mailing of the international search report

29. 04. 98

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Osborne, H

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/12347

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 318 245 A (ML TECHNOLOGY VENTURES) 31 May 1989 see page 5, line 14 - line 29 ---	1-299, 324-421
A	WO 90 14442 A (GENE TRAK SYSTEMS) 29 November 1990 see page 21 - page 24 ---	1-299, 324-421
A	LENDELL L ET AL: "Characterization of fully modified oligoribonucleotide hetero- and homoduplex hybridization and nuclease activity" NUCLEIC ACIDS RESEARCH, vol. 23, no. 11, 11 June 1995, pages 2019-24, XP002048574 see the whole document ---	1-299, 324-421
P,A	EP 0 742 287 A (MCGALL GLENN H ;MIYADA CHARLES G (US); CRONIN MAUREEN T (US); TAN) 13 November 1996 see page 11, line 39 - page 13, line 15 ---	1-299, 324-421
A	US 4 797 355 A (STABINSKY YITZHAK) 10 January 1989 see the whole document ---	1-299, 312-319 300-307
X	EP 0 421 725 A (GENE TRAK SYSTEMS) 10 April 1991 see page 6; figure 1 ---	300-307 312-319
X	WO 96 06950 A (CHIRON CORP) 7 March 1996 see figure 1 ---	300-307 312-319
A	US 4 925 785 A (WANG CHANG-NING J ET AL) 15 May 1990 see figures 10,11 ---	308-311, 320-323
A	WO 95 22623 A (LANDEGREN ULF ;KWIATKOWSKI MAREK (SE)) 24 August 1995 see figure 1 ---	308-311, 320-323
A	WO 90 12116 A (GENE TRAK SYSTEMS) 18 October 1990 ---	
A	WO 91 08480 A (UNIV LELAND STANFORD JUNIOR) 13 June 1991 -----	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 97/ 12347

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. Claims 1-299, 324-421: Method for detecting and amplifying nucleic acid sequences using modified oligonucleotides having increased target specific Tm.
2. Claims 300-323: Methods for detecting a nucleic acid target in a sample using various hybridisation assay formats.

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest
- ☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. Appl. No.

PCT/US 97/12347

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9532305 A	30-11-95	AU 2522095 A EP 0760008 A JP 10500310 T	18-12-95 05-03-97 13-01-98
WO 9514706 A	01-06-95	US 5700922 A AU 1186095 A CA 2177357 A EP 0734391 A JP 9500394 T	23-12-97 13-06-95 01-06-95 02-10-96 14-01-97
EP 0415901 A	06-03-91	US 5134066 A CA 2024182 A JP 1967673 C JP 3098599 A JP 6102676 B US 5359053 A	28-07-92 01-03-91 18-09-95 24-04-91 14-12-94 25-10-94
WO 9313121 A	08-07-93	AU 669353 B AU 3427593 A CA 2126691 A EP 0618925 A JP 6511155 T US 5700922 A US 5623065 A	06-06-96 28-07-93 08-07-93 12-10-94 15-12-94 23-12-97 22-04-97
EP 0318245 A	31-05-89	US 5030557 A AT 106947 T AU 2611288 A CA 1319336 A DE 3850055 D DE 3850055 T DK 361289 A ES 2056115 T FI 893526 A JP 2502250 T KR 9615893 B PT 89050 A WO 8904876 A	09-07-91 15-06-94 14-06-89 22-06-93 14-07-94 29-09-94 20-09-89 01-10-94 21-07-89 26-07-90 23-11-96 01-12-88 01-06-89
WO 9014442 A	29-11-90	AT 127856 T	15-09-95

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. Application No

PCT/US 97/12347

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9014442 A		AU 5966990 A CA 2031490 A DE 69022362 D DE 69022362 T EP 0426843 A JP 4500316 T US 5432271 A US 5217862 A	18-12-90 25-11-90 19-10-95 09-05-96 15-05-91 23-01-92 11-07-95 08-06-93
EP 0742287 A	13-11-96	NONE	
US 4797355 A	10-01-89	NONE	
EP 0421725 A	10-04-91	AT 138105 T AU 6360690 A CA 2025178 A DE 69026995 D DE 69026995 T JP 3216200 A US 5466577 A	15-06-96 11-04-91 03-04-91 20-06-96 21-11-96 24-09-91 14-11-95
WO 9606950 A	07-03-96	US 5681702 A AU 3463195 A CA 2197901 A CZ 9700589 A EP 0778898 A FI 970803 A NO 970884 A PL 318933 A SK 25497 A	28-10-97 22-03-96 07-03-96 16-07-97 18-06-97 23-04-97 28-04-97 21-07-97 10-12-97
US 4925785 A	15-05-90	NONE	
WO 9522623 A	24-08-95	EP 0745140 A JP 9509063 T	04-12-96 16-09-97
WO 9012116 A	18-10-90	AU 5401290 A CA 2031536 A DE 69030804 D DE 69030804 T	05-11-90 06-10-90 03-07-97 06-11-97

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/US 97/12347

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9012116 A		EP 0431086 A	12-06-91
		US 5589335 A	31-12-96

WO 9108480 A	13-06-91	US 5015569 A	14-05-91
		AU 7048791 A	26-06-91
		CA 2069948 A	02-06-91
		EP 0503000 A	16-09-92
		US 5474911 A	12-12-95
